BUILDING COMPLEX COMMUNITIES TO PROMOTE INDIVIDUAL MEMBERS' PERSISTENCE AND AFFECT BEHAVIORS

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ABSTRACT

Susanna L. Harris: Building Complex Communities to Promote Individual Members' Persistence and Affect Behaviors
(Under the direction of Elizabeth A. Shank)

Faced with a growing population to feed and a lack of environmentally friendly agricultural interventions, researchers are turning to microbes as the next solution to improve crop yield. Plant growth-promoting bacteria (PGPB) are applied directly to crops or soil to increase plant development, survival or yields. As some PGPB do not maintain colonization on plant roots, the grower must apply them multiple times to maintain any benefit. Multispecies bacterial communities often show synergistic traits, including in some cases increasing the survival or biomass of individual bacterial species. I thus hypothesized that bacterial co-inoculants could improve PGPB association with plant roots. In my dissertation, I examine root colonization dynamics of bacterial communities and analyze how co-inoculation with rhizosphere bacteria affects PGPB abundance and localization on the root.

To measure the kinetics of bacterial association with plant roots, I created a high-throughput system for hydroponic growth of plants. In contrast with the PGPB strain *Pseudomonas simiae* and bacterial isolates native to the *A. thaliana* rhizosphere, the model soil-dwelling bacterium *Bacillus subtilis* did not maintain initial colonization, mirroring a phenomenon reported in field trials of *Bacillus* PGPB interventions.

I used this system to characterize 96 fully genome-sequenced bacterial strains isolated from the *A. thaliana* rhizosphere for their ability to maintain colonization on *Arabidopsis* roots or improve the maintenance of *B. subtilis*. I characterized three strains for their effects on *B. subtilis* over a longer time course and determined their spatial localization along the seedling root. I found that these rhizobacteria also increased the maintenance of two commercially available PGPB strains of *Bacillus amyloliquefaciens*.

The long-term goal for this research is to identify functional microbial consortia that consistently associate with plant roots to improve plant health and yields. These results illustrate the utility of our system to address questions about plant-microbe interactions and may help address observed inconsistencies across different PGPB intervention studies. This line of research could ultimately help enhance the strength and reliability of agricultural microbial bioadditives, which have the potential to become viable alternatives to environmentally and economically costly agrochemicals and pesticides.

ACKNOWLEDGEMENTS

This dissertation is dedicated to those special humans who hear

“I’m not okay”

and respond with

“that’s okay, how can I help?”

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LIST OF ABBREVIATIONS AND SYMBOLS

Agtech – Agricultural Technology

A. thaliana – Arabidopsis thaliana

A. nicotinovorans – Arthrobacter nicotinovorans

B. amyloliquifaciens – Bacillus amyloliquifaciens

B. cereus – Bacillus cereus

B. licheniformis – Bacillus licheniformis

B. subtilis – Bacillus subtilis

B. thuringiensis – Bacillus thuringiensis

BSL – Biosafety Level

CFP – Cyan Fluorescent Protein

C. oceanosedimentum – Curtobacterium oceanosedimentum

ddH_20 – Deionized Distilled Water

MLS – Erythromycin-Lincomycin

GOI – Genes of Interest

GMO – Genetically Modified Organism

g – Grams

Hpt – Hours Post-Transfer

HCL – Hydrochloric Acid

h or hr – hour

ISR – Induced Systemic Resistance

LB – Lysogeny Broth

$MgCl_2$ – Magnesium Chloride

MALDI – Matrix Assisted Laser Desorption/Ionization

M. oleivorans – *Microbacterium oleivorans*

MES – [2-(N-morpholino)ethanesulfonic acid]

MS – Murashige and Skoog

p – P-value

µg – Micrograms

µL – Microliters

mL – Milligrams

mL – Milliliters

nm – Nanometers

OD_{600} – Optical Density at the 600 nanometer wavelength

PGPB – Plant Growth-Promoting Bacteria

PGPR – Plant Growth-Promoting Rhizobacteria

PPE – Personal Protective Equipment

P. fluorescens – *Pseudomonas fluorescens*

P. putida – *Pseudomonas putida*

P. simiae – *Pseudomonas simiae*

qPCR – Quantitative Polymerase Chain Reaction

rRNA – Ribosomal Ribonucleic Acid

Rpm – Rotations per minute

s – seconds

sp. – Species (singular)

spp. – Species (plural)

URM – Underrepresented Minority

VOC – Volatile Organic Compound

X-Gal – 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside

YFP – Yellow Fluorescent Protein

CHAPTER 1: INTRODUCTION

1.1. Harnessing the Phytobiome

We are facing an impending crisis of food security. Studies estimate that agricultural yields will fail to meet the needs of the global population by the year 2050[1]. In addition to the ever-growing human population, climate change due to human activities threatens to destabilize our current farming systems. To address these concerns, we must quickly identify methods for increasing food production while minimizing the negative environmental impact of using harsh agrochemicals.

Traditional farming approaches have focused on breeding plants for traits like increased yield and time to harvest, adaptation of the environment through building irrigation systems, and application of synthetic fertilizers and chemicals to protect against pests and pathogens[2]. While up to this point, these measures have greatly increased our ability to produce food, most of these applications originating from the Green Revolution are not optimal for improvement[3,4]. For example, plant breeding for desired traits requires creating and testing new crops over multiple growth cycles, which can take years from initial experimentation to the identification of a substantially better product[2]. Scientists can shortcut this process by directly modifying the genome of the plant[2]; however, insufficient data around the environmental impacts of GMO crops[5] and consumer concerns about GMO foods has slowed the use of these technologies for implementation in commercial agriculture[6]. Although effective in the shorter term, environmental studies show that intensive field management and applications of agrochemicals

harms the environment by depleting it of resources and reducing biological diversity. Runoff from these chemicals, which is increased by certain irrigation and planning systems, has negatively impacted waterways and soil systems around the world[7]. Scientists are therefore increasingly focusing on how to improve plant health without the needs for such measures.

Instead, to improve crop yield and food production, researchers hope to identify natural promoters of plant health by studying the biological interactions occurring in the phytosphere. The phytosphere comprises all environmental components of the ecosystem in which a plant is grown; this includes the bacteria[8], fungi[9], archaea[10], and animals[11] living on and around the plant, as well as the abiotic factors of the soil substrate in which they live. Phytobiomes (the collection of organisms found within a phytosphere) are shaped by interactions within their ecosystem; living organisms interact with each other, as well as with their environment, and their behaviors are determined by a complex interplay of chemical and physical signals. Maintaining the balance of these interkingdom interactions is important for the survival of all members of the ecosystem, since small perturbations can lead to the plant's destruction by microbial pathogens[12]. Plants that are abundant producers under optimal conditions can fail as crops if their growth conditions shift towards an imbalanced state. By promoting healthy phytobiomes, scientists and farmers could better address food production concerns while maintaining the stability of the phytosphere and the health of the soil for long-term crop production.

1.2. Plant Growth-Promoting Bacteria

Certain species of plant-associated bacteria can, when used as field additives, increase plant growth, resilience to stressors, and resistance to pathogens. Such bacteria, collectively referred to as Plant-Growth-Promoting Bacteria (PGPB), have been the focus of industrial and

academic researchers alike. Species of *Bacillus* are commonly described as PGPB and have become attractive targets for development into agricultural additives due to their wide host ranges and their ability to form hardy, dormant endospores that can be safely and stably stored for prolonged periods of time. One concern of using PGPB-based interventions is that some of these bacteria do not maintain their original levels of colonization on plant roots, and the treatments may thus lose efficacy over time. For my dissertation I have investigated the hypothesis that bacterial co-inoculants could improve the association of PGPB with plant roots; specifically, I have examined the dynamics of root-associated bacterial communities and analyzed how co-inoculation with rhizosphere isolates affects PGPB abundance and localization on the *A. thaliana* root

1.3. *Bacillus* Species as Plant Growth Promoting Bacteria (PGPB)

Members of the genus *Bacillus* are Gram-positive facultative anaerobes that can form hardy dormant endospores (here simply called spores) that allow them to survive restrictive conditions like high heat and low levels of hydration or nutrient availability[13,14]. *Bacillus* species have been isolated from myriad environments, from the depths of the oceans to within mammalian hosts and even from surfaces of the International Space Station[15–17]. In addition to the ability of *Bacillus* species to affect plant health and alter the diversity and stability of endogenous soil microbiomes [13,14,18–21], the broad ability of this genus to produce specialized metabolites has been leveraged to generate antibiotics and bioactive molecules for use in human health[17,22,23]. These applications have led to new *Bacillus* isolates being continually sought after and characterized for their agricultural and industrial potential. They have also led to extensive

genomic sequencing of these environmental bacteria by both governmental and industrial agencies[24]: over 4,000 strains of *Bacilli* and their genomes are currently publicly available[25].

Interest in this taxon has also been driven by the genetic tractability and robust biofilm formation of several species of *Bacillus subtilis* under both *in vitro* and *in vivo* conditions[26,27]. Laboratory strains such as *B. subtilis* 168 readily take up exogenous DNA, enabling scientists to easily modify its genome and biochemical activities, while the NCIB3610 strain of *B. subtilis* (likely an ancestor of 168) may better approximate the phenotypes of native soil bacteria while still allowing for genomic manipulation through bacteriophage transfection[28,29].

Strains of *Bacillus amyloliquifaciens* are closely related to *B. subtilis* and are some of the most extensively studied and commercially applied rhizobacteria for agricultural use[29,30]. *B. amyloliquefaciens* strains FZB42 and GB03 are the bioactive components of multiple companies' patented formulations for field additives to promote crop growth and increase yields [14,31]. Much of the appeal of *Bacillus* species as biofertilizers is that, in contrast to other well-known PGPB such as *Pseudomonas* species, the ability of *Bacillus* to form spores means they can be made into shelf-stable inoculants that can be added directly to soils or into seed mixtures as biofertilizers[17]. While dozens of different *Bacillus* strains have been identified in association with plant roots – and thus have the potential to benefit plant development – researchers have traditionally focused on characterizing only a handful of *Bacillus* species [22,26,29,30,32].

This introduction focuses on the application of *Bacillus* species for the promotion of plant growth through the inoculation of plant rhizospheres, the region directly surrounding plant roots. I will also highlight the potential benefits and limitations of *Bacillus* species as PGPB in improving crop yield along with directions for future research. Note that while species of

Paenibacillus have historically been erroneously identified as *Bacillus*, the research on these bacteria is outside the scope of this dissertation[8].

1.4. Mechanisms of Bacillus Plant Growth-Promotion

PGPB are so-named due to their observed ability to increase plant growth, development, fruiting or flowering yield, or survival[16,26]. The factors driving these phenotypes can be characterized into three overlapping categories: I) manipulation of plant chemical production or gene expression, II) direct inhibition of pathogens, and III) modulation of plant-associated microbiomes. As others have extensively covered these effects[17,26,30,33], I will only briefly summarize recent findings about how interactions with *Bacilli* affect plant health and growth with regards to these themes.

1.4.1. Manipulation of Plant Chemical Production or Gene Expression

Plants frequently experience periods of drought, variable temperatures, and disturbances of soil pH or salinity. *Bacilli* can support plant health by enhancing plants' growth and ability to withstand such environmental stressors[17]. Especially as climates change rapidly, these effects may prove useful in supporting crop growth in regions that may become otherwise restrictive to some cultivars. Specifically, some *Bacillus* species have been shown to increase water uptake by plants[26], while others spur storage of osmo-protectants like glycine or betaine, which enhance survival when there are variations in soil salt concentrations[33]. The bioactivities of *Bacillus* species can increase the availability of nutrients and minerals for plants' development and growth; for example, production and secretion of bacterial siderophores collect trace iron in the soil surrounding plant roots[8]. A single species of *Bacillus* can affect plant health through

multiple effects; for instance, not only does *Bacillus licheniformis* promote photosynthesis in wheat, but also the cytokinins secreted by this bacterium speed plant development and modulate root structure to increase water and nutrient uptake[33,34].

1.4.2. Inhibition of Pathogens

Infections by plant pathogens result in crop losses totaling over one billion US dollars annually[35]. Identifying measures to decrease pathogen infection and plant death without further use of chemical pesticides and antibiotics has come to the forefront of bioadditive research[13,17,27]; bioadditives are technically considered biologically derived additives, either bacteria themselves or their products. *Bacilli* can increase plant resistance to microbial pathogens, both through direct antagonism of the invading organisms and by changing plants' physiology through biochemical characteristics to better resist infection[36,37]. *Bacilli* can directly and indirectly inhibit bacterial, fungal, viral, and even microscopic eukaryotic pathogens[38]. Direct antagonism of pathogenic microbes is often mediated through the production of antibiotics and antimicrobials, and *Bacilli* dedicate substantial genomic space to encoding such specialized metabolites[14,39–41]. *Bacillus* species can also directly outcompete other microbes for resources during colonization and thus dominate the plant niche[42].

Bacillus colonization of the rhizosphere can increase plants' ability to defend themselves against foliar pathogens; this phenomenon is called Induced Systemic Resistance (ISR)[31]. These long-range impacts of *Bacillus* root colonization have been attributed to the production of bacterial volatile organic compounds (VOC) and cytokinins that induce immunogenic changes in the plant though hormone signaling; plant immune pathways controlled by auxin, salicylic acid, and jasmonate-ethylene are differentially regulated by the presence of natural isolates and

laboratory strains of *Bacillus*[28,39,40]. This protection primarily occurs through priming of defense responses both at multiple levels of plant physiology, from regulation of transcription to changing post-transcriptional mechanisms[17]. These effects are promising and have been observed to reduce the impact of pathogens on crops, including staple agricultural plants like tomatoes, tobacco, lettuce, and cereals[24,26,43,44]. Other *Bacillus* compounds such as fungicins, surfactins, acetoin, 2,3-butanediol, and various iturins have also been shown to activate these immune pathways[37,40,45,46]. These chemicals are often produced during *Bacillus* biofilm formation, indicating that the ability of these strains to generate anti-pathogen molecules may depend on their environment and developmental stage.

1.4.3. Modulations of Plant-Associated Microbiomes

One of the potential benefits of using bioadditives rather than agrochemicals is the hypothesis that adding microbes to fields will be less disruptive to the endogenous field microbiome than traditional agrochemicals[17,41]. The research supporting this idea is still contentious, however. In some cases, small and sustainable shifts in microbial community composition have been reported and attributed to the presence and activities of *Bacilli*[21,32,44]. One study demonstrated that the addition of *Bacillus* species to broccoli roots specifically altered the composition of the endophytic microbiome[20]. In contrast, other researchers have proposed that these shifts might instead be driven by factors such as pathogen presence, plant age, and the crop system itself[47,48], or due to the ability of *Bacilli* to shift the microbiome composition to be more protective as a whole rather than due to the activity of the PGPB directly[19].

1.4.4. Required Properties for Producing Plant-Beneficial Effects

Some factors that support beneficial *Bacillus*-plant associations are generally conserved across bacterial and plant species[17].

1.4.5. Chemotaxis

For bacteria to successfully colonize a plant root, they must first locate and move towards the root surface, or rhizoplane. *Bacillus* cells containing mutated chemotaxis machinery (that are thus unable to move towards the gradient of root exudates nutrients such as sugars and amino acids) are defective in initiating rhizoplane colonization[36].

1.4.6. Biofilm Formation

Following contact with the root, *Bacilli* switch from their motile state to a stationary (biofilm) state and attach to external root cells. This change from a planktonic form to a biofilm-producing form requires a massive overhaul of genetic regulation[15]. Biofilms are thought to provide benefits to the plant[20] as well as to allow the bacteria to survive and maintain on the plant root by protecting them from drought and salinity stress and shielding them from physical disturbances[19]. *Bacillus* strains mutated to no longer produce biofilm structural components or unable to sense the environmental signals that stimulate the expression of biofilm genes are deficient in plant association[26,37,49]. Genes encoding biofilm production are conserved across many *Bacillus* species of PGPB[24] and both *B. subtilis* and *B. amyloliquefaciens* biofilms have been found on the rhizoplane of crops grown in soil, demonstrating that they form biofilms on plant roots *in situ*[15,50].

1.4.7. Metabolite Production

Within these biofilms, bacteria produce antibiotic compounds and other molecules that can be perceived as signals by neighboring microbes and plants. These compounds include VOCs. VOCs have been shown both to directly inhibit pathogens as well as to elicit ISR, a plant immune response that increases resistance to pathogens in both the rhizosphere and the above-ground phytosphere [26,38,51]. For example, production of 2,3-butanediol by *B. subtilis* inhibits fungal plant pathogens whether or not the bacterium is present; interestingly, these volatiles do not affect *Pseudomonas protegens*, another known PGPB[52]. Non-volatile metabolites also impact rhizosphere colonization by PGPB. For instance, fengycins have been found to be important in restricting fungal disease on tomatoes, as well as in bananas in pots[40]. *B. subtilis* and *B. thuringiensis* colonization is enhanced by the production of specialized metabolites[22]; mutations that increase the production of specialized metabolites increase the capacity for controlling pathogens[29]. Catalases, lipopeptides, and acetoin are also produced in the biofilm stage of *Bacillus* growth, all of which have been found to inhibit plant pathogen proliferation[45,53,54].

The direct effects of specialized metabolites on PGPB colonization can be difficult to determine; effects mediated by the chemicals themselves often can't be separated from the effects of direct bacterial interactions with the plants. Nevertheless, researchers have used creative approaches even in open-soil systems (Fig. 1.1) to demonstrate that VOCs are the causative component of ISR[51].

1.4.8. Additional Influences

It is important to note that these phenotypic behaviors that appear essential for plant attachment can also be modulated by interactions with the plant host and other rhizosphere

microbes. In addition, the temperature, hydration levels, pH, and physical structure of the surrounding environment can impact *Bacillus* cellular phenotypes, as can the neighboring members of the microbiota[55]. Over time, small molecules are exchanged, nutrients used and produced, and conditions change; this complex interkingdom relationship between biological organisms entails innumerable possible variables, and reductionist experiments can only include a limited number of these factors. For instance, the bacterial production of surfactin, an essential molecule for colonization, is increased in the presence of plant roots and its exudates[39]. Even field studies only represent a subset of possible conditions. These caveats are important when considering the experimental conditions of studies designed to reveal the relationships between plant hosts and *Bacillus* species.

1.5. Colonization of the Rhizosphere in Gnotobiotic Systems

Many plant hosts can be cured of microbes to become gnotobiotic, often through seed sterilization or successive breeding to select for plants without endophytic bacteria. These simplified plant systems can then be associated with individual microbes whose behaviors, including initial colonization, biofilm formation, and chemical production, can be studied under different environmental conditions. These highly-reductionist approaches have been essential for teasing apart interactions between plant hosts and *Bacillus* species. Examples of different gnotobiotic experimental systems, including those conducted in non-sterile environments, are shown in Fig. 1.1.

1.5.1. Hydroponic Growth

Hydroponic systems (Fig. 1.1A, 1.1B) have been developed to grow plants in controlled, liquid-only environments, whether or not these plants are natively found growing in water. Gnotobiotic systems have been developed to study the hydroponic colonization (such as biofilm formation) and early few days of maintenance by rhizobacteria[56–58]. These experiments have underscored the importance of the chemotactic sensory and motility machinery for initiating colonization, and how these processes are shaped by the presence and activity of the host plant[24,36]. For example, cucumber root exudates affect *B. amyloliquefaciens* chemotaxis[52]; while bacterial intracellular signaling through cyclic-di-AMP affects biofilm formation in *B. subtilis*, thus impacting its ability to effectively colonize the rhizoplane[59]. One advantage of the hydroponic design is that these exudates can easily be collected for further characterization[52].

Studying how *Bacilli* interact with the roots of hydroponically-grown plant systems can be performed both with laboratory model organisms and agricultural crops like tomato, tobacco, cucumber, and pepper species[8,24,37,50,60]. While many studies have focused on monoculture association of bacteria with plants, hydroponic assays can also easily be used to study interactions between rhizosphere members or the direct interactions between *Bacillus* PGPB and plant pathogens[24,37]. Live-cell imaging using non-destructive approaches like confocal fluorescence microscopy allow researchers to monitor the spatiotemporal characteristics of these host-microbe and microbe-microbe interactions over time[16,56]. Despite these advantages of hydroponic systems, growth in liquid is not suitable for most plants long-term and the findings from these experimental conditions may not reflect the interkingdom interactions occurring in field soils. Furthermore, many hydroponic studies add simple sugar carbon sources such as

glycerol and sucrose; since bulk soil is typically nutrient-limited, these additions should be noted when interpreting results from hydroponic or other laboratory assays that include them[27,36,60].

1.5.2. Agar-plate-based Growth

Both bacteria and plants are routinely cultivated separately on defined agar medium (Fig. 1.1C, 1.1D). The *Bacilli* and plants are typically independently cultured under growth conditions specifically suited to them, such as with increased carbon (for microbes) or increased nitrogen and phosphate (for plants). The challenges of satisfying the growth conditions of both bacteria and plants can be overcome through the use of plant-growth-medium agar supplemented with simple sugar carbons or 'split' plates that allow each organism to grow on its preferred substrates but still participate in VOC-mediated signaling[51,61]. Split-plate assays allowed scientists to identify that inhibition of fungi by *B. subtilis* was partially mediated through production of the VOC 2,3-butanediol, since the plastic barrier ensured the microbes were not in direct contact but could still pass VOCs between them[52].

As with the hydroponic-based systems described above, plants and their associated *Bacilli* can be imaged after growth on agar; a benefit of agar-based systems is that the roots often do not need to be disturbed before photographic or microscopic imaging, allowing researchers to collect data about branching and elongation patterns more easily[27]. This also enables the use of biochemical analysis, such as Matrix Assisted Laser Desorption/Ionization (MALDI) mass spectrometry, to measure the chemical signals generated by different portions of the root[14]. A restriction of agar-based systems, however, is that direct associations are studied by proximal movement towards plant roots on agar (a non-natural surface) or through direct inoculation of

part of the root, eliminating our ability to project results to interactions occurring in a three-dimensional environments like soil[38].

Agar-based assays can be modified to include mixed microbial inoculants rather than single bacterial species as well as plant root exudates (in addition or in place of plant roots themselves). For example, three bacteria repeatedly co-isolated from the soybean rhizosphere (*B. cereus, Flavobacterium johnsoniae*, and *Pseudomonas koreensis*) were interrogated on agar, demonstrating a number of pairwise and emergent coculture interactions in terms of their colony expansion and biofilm formation[62]. In another example, by growing both plants and bacteria on the same agar, researchers showed that *B. subtilis* was able to outcompete *Serratia plymuthica* to reduce rhizosphere infection by this pathogen[39]. By adding root exudates into bacterial growth agar, the presence of plant-derived chemicals were shown to affect the interactions between *B. cereus* and other plant commensal bacteria[63].

Again, it is important to note that these plate-based interspecies interactions may not translate into more real-world conditions; for instance, measurements of the inhibition of potato pathogens by *Bacillus* species on agar differed when compared to growth on potato tubers[42]. Nevertheless, given the complexity of soil and field sites, these simplified systems have enabled a large number of mechanistic and controlled studies to be performed. In addition, since crops can potentially be pre-colonized by PGPB prior to introduction in a field, the transfer of plants from agar medium or liquids to other conditions or soils is increasingly being studied[58,64]. These findings will be discussed in more detail under the Maintenance section and may enable more stable translation of laboratory findings into the fields.

1.5.3. Microcosm Growth

The physical structure of environments can affect the phenotypes of both microbes and plants; thus, the interactions between a microbiome and its host may similarly be affected. Multiple systems have been designed to model plant-microbe interactions while still allowing non-destructive monitoring of these processes over time. Microcosms that include ports for flow of liquids containing chemicals, nutrients, and salts can help scientists understand how microbial species and combinations of bacteria colonize and survive on plant roots during changing environmental conditions[26,29].

Optically transparent substrates, such as the pseudo-soil Nafion, provide a soil-like structure in which to observe colonization of the rhizosphere in an environment where plants display extensive branching and elongation patterns reminiscent of those in native soils[65,66]. As this substrate is transparent when saturated with aqueous solutions, the microcosms can be subjected to confocal microscopy for ongoing observation. Multiple bacterial species can be co-inoculated into these pseudo-soil systems, as was done to study how *P. fluorescens* and *B. licheniformis* interact to positively increase biofilm production[34]. In addition to these microcosms, EcoFAB, a recently formed cross-disciplinary initiative, is focused on stimulating discussions and collaborations by scientists interested in probing ecosystem processes through controlled model ecosystems[67]; this approach may stimulate new and unified experimental systems to ask about plant-microbe interactions.

1.5.4. Closed-soil Systems

The composition of the rhizosphere microbiome can be influenced not only by plant species but also by the type of soil the plants are grown in[68]. Using sterilized soils or substrates

that approximate soil (like clay or sand) are therefore especially useful in conducting closed-system assays: those in which only specific microorganisms are introduced. In these sterile, closed systems, the structure and chemical composition of the soil is more complex than in synthetic systems, but more simplified than native soils.

Scientists have developed multiple ways to rid natural soils of their endogenous microbes, with each technique having pros and cons. One early approach of autoclaving thin layers of soil to reach sterility is accessible to most research laboratories, but the autoclaving process both destroys some natural soil biochemicals while also producing new compounds toxic to some microbes[69]. To address this toxicity, scientists have recently developed a time-intensive hot-water rinsing technique to reduce chemical alteration of the soil while still ridding the soil (peat in this instance) of endogenous organisms[70]. Furthermore, many fungi and some bacteria form spores that are recalcitrant to autoclaving, in part because the soil structure itself can reduce local temperatures and pressure gradients, reducing the efficacy of autoclaving[70]. To minimize these concerns, gamma radiation has been applied to thin sheets of soil, either alone or in conjunction with autoclaving[69]. However, few researchers have access to gamma radiation sources, limiting the adoption of this effective approach.

As these sterilization processes for soil are time-intensive and can produce unwanted effects, the use of sterile calcined clay and sand has been adopted by some researchers. These substrates can be more effectively sterilized by autoclaving, especially in terms of ridding the system of spore-forming fungi. Further, most of the particles within these substrates are of approximately the same size and composition, reducing the heterogeneity of the growth substrate. *Bacillus subtilis* forms biofilms on the surfaces of different soil clay minerals depending on their chemical composition, and insecticidal proteins from *B. thuringiensis* can

adhere to clay particles, suggesting that these systems can affect *Bacillus* phenotypes[71,72]; how these behaviors replicate those in more native soils is unclear.

One disadvantage of these sterile-soil approaches is that the opacity and spectral properties of these substrates inhibit the use of most imaging techniques for visualizing bacteria within the rhizosphere; even after multiple rinses, particles of soil remaining adhered to plant roots often exhibit fluorescence or refract light, restricting the use of fluorescence microscopy. X-ray imaging allows researchers to monitor spatial changes in soil and the plant root morphology, but is unable to measure microbial localization or behaviors[69,73]. One approach that has been used is to introduce small numbers of native soil particles into sterilized microcosms to better approximate interactions in complex structural environments while still enabling visualization[34].

1.5.5. Open-soil Systems

When compared to gnotobiotic systems, experimental systems that are open to environmental influences and microbes (such as greenhouse assays or field experiments) provide both positives and negatives. Arguably the largest benefit of these open-soil systems is their incorporation of the natural conditions in which crops grow. These systems can also be designed to enable study of plants over the longer growth cycles relevant to commercial cultivation. In the case of greenhouse experiments, scientists can dictate the exact temperatures, light settings, soil types and properties, and hydration levels for plant growth. In contrast, in field settings only a small number of potential variables can be controlled by human intervention: the environment is largely determined by the weather and pre-existing endogenous microbes and soil properties, whether conducted in uncultivated areas or in industrial farm fields. Nevertheless, both

greenhouse and field settings can be manipulated by the addition of PGPB inoculants, such as *Bacillus* species, as well as affected by the addition of fertilizers, chemicals, water, and pathogens.

In open-soil systems, uncontrolled variables can affect both the phenotypes of the plants and their associated microbes. Variations between biological and technical replicates must be accounted for when interpreting findings, as the weather across growth seasons or the specific location in a field can affect local growth environments. These differences can be partially mitigated through the randomization of plantings and the separation of samples to reduce cross contamination. Increasing the total sample size or replication across growing seasons can lend statistical significance to findings but also requires more space, time, and money per experiment. Ecological studies can provide references for best practices[48].

To increase throughput of open-soil systems and account for variability, seeds or seedlings can be pre-inoculated with *Bacillus* species. During this process, seedlings can be collected and analyzed to measure the pre-colonization efficiency of the PGPB inoculation as well as the evenness of plant size distribution. This may prove particularly important since it appears that *B. subtilis* inoculation in field settings results in differences in plant size; this size variability would be detrimental to harvesting procedures, even if the overall average size of the crops were increased[20]. Whether this can be ameliorated or accounted for by pre-inoculating and selecting consistently sized seedlings for the field remains to be seen.

Alternatively, bacteria can be directly added to the plants in the field, either alone or following pre-inoculation of seeds and seedlings. For example, *B. amyloliquefaciens* FZB42 added to seeds of lettuce directly and then also added to the fields in which the seeds were sown resulted in measurable rhizosphere colonization of the lettuce plants[74]. However, another group

reported conflicting results, indicating that *B. amyloliquefaciens* failed to colonize lettuce for more than a few days, although it did have sustained effects on the lettuce rhizobiome[51]. Yet another study found that *B. amyloliquefaciens* spores applied directly to soil could colonize the rhizosphere of lettuce both in the field and in greenhouse conditions[74]. Maintaining PGPB colonization following field inoculation or after transfer to different growth environments will be discussed below in the Maintenance and Microbiome Effects section.

1.6. Selection of Individual and Combinations of PGPB

In addition to identifying and applying single PGPB, many studies are driven by the desire to identify new bioactive strains of *Bacilli* or to understand how combinations of strains may impact plants. Synergistic effects between different phyla of PGPB have also been demonstrated: chickpea plants inoculated with both *P. putida* and *B. amyloliquefaciens* in a greenhouse setting showed increased drought tolerance compared to the effects of either inoculum alone[75]. One advantage of using non-sterile soils is that these systems enable researchers to screen for wild PGPB isolates. In a study of heat-stressed wheat plants, four bacterial strains (of *Pseudomonas brassicacearum, B. thuringiensis, B. cereus, and B. subtilis*) were identified that increase drought tolerance due to their production of catalases and other specialized metabolites[53]. Similarly, isolates of *B. subtilis* taken from the rhizosphere of healthy tomato plants were found to increase the host's resistance against *Ralstonia solanacearum* due to conserved biofilm production genes[24].

In addition to single PGPB, simplified communities have been identified that reproducibly and sustainably colonize plants, such as a community of bacteria originally derived from corn[76]. Scientists are also focusing on cultivating healthy microbiomes that include strains

of PGPB *Bacilli*. Some bacterial combinations exhibit additive effects on their community members in terms of proliferation and biofilm production, both of which are required behaviors for effective application of *Bacilli* as PGPB[19,20,53]. In another example, one strain of *B. cereus* was selected for its ability to co-exist and promote growth of commensal rhizobacteria, suggesting possible routes for discovery of PGPB able to coexist with members of endogenous soil microbiome[63]. Finally, the THOR microbiome was designed around *B. cereus* and the "hitchhiking" bacteria often co-isolated with it from the soybean rhizosphere; these isolates increase biofilm production by both *B. cereus* and a *Pseudomonas* species[62].

1.7. Maintenance and Effects on the Microbiome

While colonization efficiency and the effects immediately following colonization are important for effects on the host plant as discussed above, sustained *maintenance* of PGPB and their systemic effects on the plant microbiome are likely even more crucial for the long-term utility of PGPB as bioadditives – particularly regarding their likelihood of replacing commercial chemicals and fertilizers. (For the purpose of this dissertation, I consider "maintenance" to mean that the inoculated strain can be identified in association with the host plant after either two weeks in a single environment or at least three days following transfer of the plant to a new growth condition.) Maintenance of bacterial presence in the rhizosphere can be monitored by differential selective plating or by sequence-based approaches. In either case, the ability to reliably identify and quantify the exact *Bacillus* strain that was originally inoculated is critical, although often overlooked: genera-level sequencing or simply plating for *Bacilli* with no additional strain-specific selection step severely limits meaningful interpretation of results, as *Bacilli* are some of the most abundant species found in both bulk soil and in the rhizosphere[77].

Assessment of maintenance by PGPB *Bacillus* species through these approaches has indicated that some bacteria show reproducible long-term associations with plants; a selected set of recent articles specifically focused on the maintenance of inoculated *Bacilli* is compiled in Table 1.1. In brief, maintenance has been observed in both gnotobiotic and open systems, from greenhouses to field trials[19–21,34,40,44,48,51,52,74(p42),78–80]. Maintenance studies using plant species from *A. thaliana* to major crops such as wheat to soybeans have explored host plant stresses induced by pathogens or environmental conditions; this shows general trends do exist across plant species but that even small variations in plant genotype or growth setting can significantly impact plant development.

Despite the encouraging studies summarized in Table 1.1, many *Bacillus*-based biofertilizers fail to produce robust beneficial results in the field, even when laboratory or greenhouse trials indicate their potential as PGPB[23]. Thus, while bacterial additives are less costly to produce and apply than other fertilizers, it is unclear whether PGPB will consistently show comparable effects on crop yield increases and over what timespans; the long-term effects of PGPB application may depend on whether these microbes are maintained on plants and how they impact the overall plant and soil microbiomes[20,79].

1.8. Effects of *Bacillus* PGPB on Field Microbiomes

Bioadditives such as *Bacillus* strains are often marketed as not only being more sustainable than chemical fertilizers or antibiotics, but also causing a smaller shift to the composition of the plants' or fields' microbiomes[19,21,47,79]. It is known that chemical treatments decrease soil microbiome diversity both in bulk soil and in the rhizosphere, and that the evenness and complexity of the surrounding microbiota can then continue to affect soil microbiome

balance and plant health[21,81]. Multiple studies have shown that introduction of PGPB *Bacilli* result in measurable changes in the plant and surrounding soil microbiomes[19,20,44,64,79]. These shifts in microbiome composition at both the species and genera levels can occur even when the PGPB inoculant is not maintained at detectable levels[20,79]. It is worth noting that these effects on microbiome composition are difficult to separate from those due to differences in plant age, plant development, presence of a pathogen, or other impacts the PGPB might have on the plant or its pathogen resistance[19,44,74]. Other studies report minimal or no changes to the endogenous microbial communities upon PGPB treatment, especially as compared to the substantial shifts observed following the addition of chemicals like fungicides[19,21].

1.8.1. Concerns about Using *Bacillus* Species as PGPB

As noted above, one concern regarding the use of *Bacillus* PGPBs is that *Bacillus* inoculation into fields has been observed to increase the size distribution of crop plants, possibly due to varying levels of maintenance on individual plants[82]. These differences in maintenance could possibly underly relatively large variances within datasets, whether between biological or technical replicates; however, most studies do not compare PGPB presence on individual plants with that specific plant's growth phenotype; data is invariably only examined in aggregate. Additionally, I am not aware of studies which have directly assessed whether measurable growth promotion following inoculation with spores of *Bacilli* correlate with the germination rate of the spores in the field, although the presence of these species can be measured for at least five weeks following introduction into a field[48,74]. These data may be important for dissecting whether the *Bacilli* are directly inducing effects on the plants or if the presence of the (even un-germinated)

spores themselves change the native microbiome such that the composition or activity of the microbiota elicits the beneficial effects[48].

Finally, some concerns have been raised about *Bacillus*-based crop treatments since in some cases *Bacillus* species appear to increase the growth and proliferation of pathogenic microbes, as demonstrated with the potato pathogen *Fusarium coeruleum*[42]. Again, understanding the complex layers of interactions between soils, plants, and bacteria that occur in response to adding PGPB to fields will require significantly more work before these approaches become consistent and predictable.

1.9. Introduction to the Dissertation

Researchers are turning to using plant growth-promoting bacteria (PGPB) to increase plant development or survival rates[83]; if adopted in place of current agricultural techniques, PGPB treatments would allow farmers to reduce their use of environmentally and economically costly chemicals. The adoption of PGPB based interventions has been slowed due to their varying levels of efficacy, which may be partly due to the loss of initial bacterial association with the plant hosts[74]. Understanding the mechanisms underlying the loss of association and defining potential means by which to improve beneficial outcomes would help researchers develop more effective agricultural interventions. As multispecies microbial communities can promote the growth and development of their individual members[76,84], it is possible that bacterial communities in the rhizosphere affect interactions of PGPB with the host plant. Using a hydroponic growth system developed in this dissertation work[58], I investigated root colonization dynamics of bacterial communities and analyzed how bacterial cocolonization affects PGPB

associations with roots of *A. thaliana* seedlings. My thesis concentrated on testing the hypothesis that bacterial co-inoculants could improve PGPB association with plant roots.

To test differences in bacterial strains' abilities to maintain initial colonization of a plant root, I created a novel experimental system for hydroponic growth *A. thaliana*[58]. Using this system, I found that the PGPB strain *Pseudomonas simiae* maintained initial colonization of *A. thaliana* seedling roots, while the PGPB strain *B. subtilis* was unable to do so. After screening a large collection of bacterial isolates native to the *A. thaliana* rhizosphere, I determined that 61% of the strains were able to maintain colonization on their own, and that five of the 96 screened strains increased the colonization and maintenance of *B. subtilis* when co-inoculated. Of these, I characterized prolonged maintenance and spatial localization of *B. subtilis* on the seedling root when in coculture with three of these strains, ultimately finding that each strain and a combination of all three also increased the maintenance of two commercially available PGPB strains of *Bacillus amyloliquefaciens*.

The objective of my thesis was to identify conditions under which PGPB species would better colonize or maintain association with the roots of a host plant. I hypothesized that rhizosphere-dwelling bacteria could affect PGPB colonization, and that some strains might increase these or stabilize associations with the plant; my research focused on building methods to test this hypothesis and measuring the effects of rhizosphere isolates' presence on root colonization by PGPB species of *Bacillus*.

1.10. Tables and Figures

Table 1.1. Select findings from studies on *Bacillus* spp. colonization and maintenance under different experimental conditions.

Maintenance is defined by sustained association of the species for over two weeks in any system or over three days after transfer to a new environment.

Ref#	Bacillus	Plant (sp?)	System	Maintenance	Measurement	PGPB & Microbiome effect
7	18 natural isolates	Pepper	Open pots of sand mixed with different soil	Disease incidence at 7 days, rhizomicrobiome samplings at 27, 31, 35, 38 days	High-throughput 16S rRNA gene sequencing	Inhibition of P. capsici in organic soil over integrated or conventional farming, significant shifts in the microbiome among treatments
63	*B. amyloliquefaciens*	Lettuce	Open pots of sand; Field	Inoculated pre- and post-transfer to fields	CFU on selective agar including at time of transfer	Protection against R. solani, no effects on field microbiome
33	*B. amyloliquefaciens*	Lettuce	Open pots of sand	Inoculated pre- and post-transfer to fields for 2 and 4 week sampling	454-pyrosequencing of community, unclear for specific inoculants	Plants chosen by apparent R. solani bottom rot (some undetermined), strong effects on composition/diversity of microbiome by stratification of diseased vs non
37	*B. amyloliquefaciens*	Lettuce	Field	Inoculated pre- and post-transfer to fields for 0,2 and 5 week sampling	Whole metagenome shotgun library sequencing via MiSeq Illumina, check fo FZB42 genomic sequences	Only marginal differences between the rhizomicrobiome composition of treated vs. untreated plants
9	*B. amyloliquefaciens*	Soybean	Sterile vermiculite; open pots of soil	Colonization up to 30 day microbiome analysis performed after 60 days	CFU on seeds at time 0 and on plants 15 and 30 days after sowing, on LB plates with no selection	*B. amyloliquefaciens* treatment had a lower effect on the soil microbiome than addition of fungicides
8	*B. amyloliquefaciens* *B. cereus* *B. subtilis*	Broccoli	Field	Sampling at 30 days after sowing of seeds	High-throughput 16S sequencing	Strong effects on endophytic microbiome composition
71	*B. amyloliquefaciens* *B. cereus* *B. subtilis*	Wild Cabbage	Open pots of soil; Field	Pre-inoculated seedlings; inoculation in field in some trials; sampling in pots at 12 weeks, sampling in field at 14 or 16 weeks	Unclear, but mentioned that smaller size of treated plants correlates to lack of colonization	Varying changes in plant size growth promotion with only some plants growing very large and others staying the same size as controls
23	*B. licheniformis*	Wheat	Sterile Microcosm	Sustained 15 days	CFU on agar with rifampicin	Increased plant growth & photosynthesis
41	*B. subtilis*	Pepper	Agar medium	WT sustained up to 28 days; mutants for less	CFU on selective agar media for mutants	Inhibition of Trichoderma sp fungus
67	*B. subtilis*	Rice	Open pots of autoclaved soil	Sustained 30 days	CFU plating (media unknown)	Increased plant size, increased concentration of chlorophyll and carotenoids
68	*B. subtilis*	Tomato	Open pots of soil	Colonization sampling up to 21 days; imaged for colonization up to 14 days	High-throughput 16s & 18s sequencing , fluorescent imaging	Minimal effects on microbiota for up to 3 days but up to 14 days' effects on eukaryota
29	*B. velensis*	Tomatoes; Banana	Open pots of soil for tomato; Fields for banana	25 days of protective effects on tomato; 30 days post-transfer of banana	unclear for specific inoculants	Inhibition of R. solanacearum and F. oxysporum
53	*B. velensis*	Pepper	Field	Stable abundance of *B. velensis* found across sampling timepoints	Bacterial 16S amplicon sequencing and fungal ITS sequencing, specific qPCR for *B. velensis* strain	Pre-colonization increased pepper fruit yield, differences between plant rhizomicrobiomes between 30, 60, and 90 days

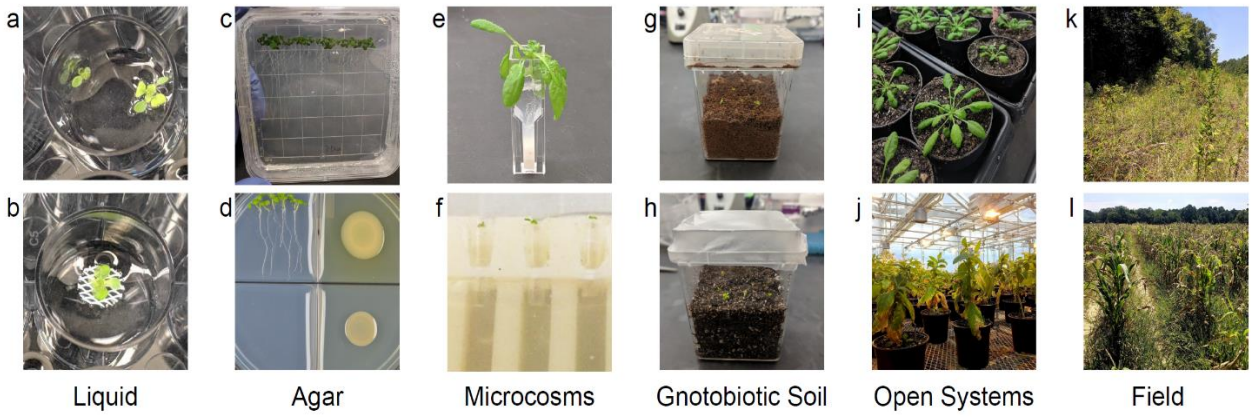

Figure 1.1. Experimental System for Examining Rhizomicrobiome Interactions.

Hydroponic systems may include plants grown in (a) or floating atop (b) liquid containing microbes. Vertical agar plates allow for visualization of root growth where plants are in direct contact with (c) or physically separated from (d) microbes. Systems can incorporate transparent granular substrates (e) or microfluidic mechanisms for liquid and nutrient flow approximate native microcosms for plant growth while allowing for visualization of interactions (f). Growth in sterilized sand (g) or soil (h) allows for introduction of plants and specific microbes in closed systems, while "open pot" systems in incubators (i) or in greenhouses (j). Associations with microbes can be further studied and surveyed with plants grown in uncultivated terrain (k) or farmland fields (l).

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CHAPTER 2: MONITORING BACTERIAL COLONIZATION AND MAINTENANCE ON *ARABIDOPSIS THALIANA* ROOTS IN A FLOATIN HYDROPONIC SYSTEM[1]

2.1. Overview

Bacteria form complex rhizosphere microbiomes shaped by interacting microbes, larger organisms, and the abiotic environment. Under laboratory conditions, rhizosphere colonization by plant growth-promoting bacteria (PGPB) can increase the health or the development of host plants relative to uncolonized plants. However, in field settings, bacterial treatments with PGPB often do not provide substantial benefits to crops. One explanation is that this may be due to loss of the PGPB during interactions with endogenous soil microbes over the lifespan of the plant. This possibility has been difficult to confirm, since most studies focus on the initial colonization rather than maintenance of PGPB within rhizosphere communities. It is hypothesized here that the assembly, coexistence, and maintenance of bacterial communities are shaped by deterministic features of the rhizosphere microenvironment, and that these interactions may impact PGPB survival in native settings.

To study these behaviors, a hydroponic plant-growth assay is optimized using *Arabidopsis thaliana* to quantify and visualize the spatial distribution of bacteria during initial colonization of plant roots and after transfer to different growth environments. This

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system's reproducibility and utility are then validated with the well-studied PGPB *Pseudomonas simiae*. To investigate how the presence of multiple bacterial species may affect colonization and maintenance dynamics on the plant root, a model community from three bacterial strains (an *Arthrobacter*, *Curtobacterium*, and *Microbacterium* species) originally isolated from the *A. thaliana* rhizosphere is constructed. It is shown that the presence of these diverse bacterial species can be measured using this hydroponic plant-maintenance assay, which provides an alternative to sequencing-based bacterial community studies. Future studies using this system may improve the understanding of bacterial behavior in multispecies plant microbiomes over time and in changing environmental conditions.

2.2. Introduction

Crop destruction by bacterial and fungal diseases results in lowered food production and can severely disrupt global stability[1]. Based on the discovery that microbes in suppressive soils are responsible for increasing plant health[2], scientists have asked whether the plant microbiome can be leveraged to support plant growth by modifying the presence and abundance of particular bacterial species[3]. Bacteria found to aid in plant growth or development are collectively termed plant growth-promoting bacteria (PGPB). More recently, studies have shifted from simply identifying potential PGPB to understanding how interkingdom interactions in the soil, around roots, or in the rhizosphere (the area directly surrounding and including the root surface) may be impacting PGPB activity[4].

Rhizosphere colonization by PGPB can increase the health or the development of host plants in response to diverse stressors relative to uncolonized plants[5]. However, results are often more variable in native soil conditions compared to those observed in the closely controlled

greenhouse and laboratory settings[6]. One hypothesis for this difference is that the growth or behavior of PGPB may be inhibited by native soil bacteria or fungi in the fields[7,8]. Beneficial effects by rhizosphere bacteria generally depend on the ability of the bacteria to 1) locate and move towards the root, 2) colonize the root through biofilm formation, and 3) interact with the host plant or pathogens via production of small molecule metabolites[7,9]. Any of these colonization behaviors may be affected by the presence and activity of neighboring microbes[10].

We designed a system to quantify and visualize these distinct bacterial colonization stages of the rhizosphere (Fig. 2.1). This approach will facilitate studies investigating why long-term PGPB maintenance is sometimes not observed following transfer of plants into new environments, such as during the planting of pre-inoculated seedlings. *Arabidopsis thaliana* was chosen as a plant model due to its extensive use in laboratory studies as well as the ample data available about its microbial interactions[11]. There are three stages in the system: 1) *A. thaliana* growth, 2) bacterial colonization, and 3) bacterial maintenance (see Fig. 2.1). Because *A. thaliana* is a terrestrial plant, it was important to ensure that it was not suffering undue water stress in the hydroponic system[12]. Inspired by the methods used by Haney et al.[13], the seedlings are grown on plastic mesh to separate the shoot from the liquid growth medium. This system does not appear to compromise the health and development of the plant host, and it improves *A. thaliana* growth in liquid[11]. As the plant shoot floats above the surface, the roots are fully exposed to colonization by bacteria inoculated into the liquid bacterial growth medium. This permits the bacteria of interest to be examined for colonization in nutrients that are most conducive to growth, while then shifting conditions to allow the plant to continue growing in a nutrient medium designed to support its growth. Both stages include steady shaking to prevent anoxia of the root[13]. Bacteria can be visualized or quantified from the plant roots following

transfer from either the colonization medium or the maintenance medium. This hydroponic system is very flexible, allowing experimental conditions and applied stresses to be easily altered depending on interests of the researchers.

This described method is important in the context of the larger body of literature about plant-microbe interactions because it provides a robust system for studying these interactions at the root surface while also being customizable to the growth preferences of different bacteria. Plant biology labs often perform plant-microbe colonization experiments on solid agar, allowing for only planar movement (if that) of bacteria while also requiring the potentially destructive manipulation of plants during subsequent transfer. In contrast, microbiology labs have frequently prioritized the health of the bacteria within their experiments, to the detriment of the plants[14,15]. These different priorities of plant- and microbiology-focused labs have historically made it difficult to compare results between these groups, since each typically optimizes experimental conditions to optimize their organism of interest[15]. The floating-mesh-plant-growth system described here prevents full plant submersion, a notable advantage to previous microbiology-oriented studies, while also temporarily optimizing the growth and survival of bacteria to facilitate colonization. Thus, the assay we present here may address concerns from both plant biologists (about over-hydration and tactile manipulation of the plant) while satisfying the criteria of microbiologists (allowing for different bacterial growth conditions and multiple species' interactions)[7]. This protocol is designed to be adaptable for use with various bacteria, plants, and environmental conditions.

2.3. Materials and Methods

The experimental setup is described for clarity and used to generate the representative results included in this report, but conditions can be modified as desired. All steps should be performed using PPE and following institutional and federal recommendations for safety, according to the BSL status of the bacteria used.

Characterization of bacteria

Determine the morphology of bacteria on the growth medium agar plate. Resuspend cells at an approximate OD_{600} = 0.5 and plate a 1 µL volume onto agar medium of choice. Add X-gal to agar plates to a final concentration of 20 mg/mL to better differentiate individual members of the specific bacterial community. Grow at 24 °C or 30 °C until colonies form, then take pictures of and notes on colony morphology.

Define the correlation between each bacterial strain's optical density and the number of CFU (colony forming units) per mL[16]. Resuspend bacteria in 1 mL of water in a 24-well plate to an approximate OD_{600} = 5, perform two-fold serial dilutions, monitor OD_{600} of all dilutions, and plate each to determine the viable CFU/mL in each sample at multiple optical densities. Determine the maximum sonication tolerance for each bacterial strain. To do this, aliquot cells into a 24-well plate containing liquid medium, reserving some cells as an unsonicated control sample. Using an ultrasonicator with a 24-tip horn attachment, apply three rounds of 12 s of sonication at 40 amp with 2 s pulses.

The use of a 24-well ultrasonicator is advised to facilitate the downstream multiplexing of processing plant samples, but if one is not available, use an ultrasonicator fitted with a microtip and perform each sample sonication independently. Always wear earmuffs rated to at least 25 NRR protection.

Perform 10-fold serial dilutions of the sonicated and unsonicated samples and spot onto agar plates. Determine whether there is a reduction in viable cells after sonication. If so, use a fresh sample and repeat sonication step using a reduced total sonication time or amplitude until the treatment has no effect on final CFU/mL[17].

Preparation of *Arabidopsis thaliana* seedlings on a plastic mesh

Create disks of the plastic mesh using a standard hole puncher. Collect the disks in a glass container with a loose cover of aluminum foil and sterilize using an autoclave set to a 20 min dry cycle[13]. Using flame-sterilized tweezers, distribute approximately 40 sterilized mesh disks in a single layer across the surface of a plant-growth-medium agar plate. Use 0.5x Murashige and Skoog (MS) salts, containing 500 mg/L of MES buffer [2-(N-morpholino)ethanesulfonic acid] and 1.5% Bacto agar, as plant growth medium, with 50 µg/mL benomyl added to the limit fungal contamination of the seedlings. Prepare axenic seeds of *A. thaliana* as previously described[17].

Place approximately 100–300 seeds each into individual centrifuge tubes in a rack and place into a resealable glass or heavy plastic container ("jar") in a fume hood. Using caution, place a beaker of 100 mL of bleach into the jar, add 3 mL of concentrated HCl to the bleach, and immediately seal the jar and allow fumes to sterilize seeds for at least 4 h.
Carefully remove the tubes of sterilized seeds from underneath the jar and seal.

Place two seeds at the center of each mesh. Seal plates with surgical tape and incubate for 2–6 days at 4 °C in darkness to vernalize seeds. To germinate and grow seedlings, place the plate agar side down in a plant growth chamber for 8–10 days under short day settings: 9 h of light at 21 °C and 15 h of dark at 18 °C (Fig. 2.1).

Colonization of plants in liquid bacterial growth medium

Add 1 mL of bacterial growth medium to each well of a sterile 24-well plate, except for media-only control wells. Use Lennox Luria Broth (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl) as the bacterial growth medium. Transfer the germinated seedlings embedded in mesh from agar plates to the liquid (Fig. 2.1). Gently peel the mesh containing two germinated seedlings up and off the agar plate using flame-sterilized forceps. Choose mesh with equally sized and undamaged seedlings. If removal from the agar is not smooth, discard that mesh and plant. Transfer one float to each well of bacterial growth liquid, root side down.

Inoculate bacteria into wells containing floating seedlings. Resuspend bacteria grown overnight on agar plates to an OD_{600} equivalent to 10^8 CFU/mL in the bacterial growth medium liquid. Add 10 µL of bacterial suspension to each well for a final concentration of 10^6 CFU bacteria per well. If preparing a mix of bacteria, resuspend each to the OD_{600} equivalent to 10^8 CFU/mL, mix in equal proportions, and add 10 µL of the final mix per well of liquid.

Seal the plate for sterile growth. Without touching the sticky side, carefully press the gas-permeable film across the plate. Ensure that each well has been individually sealed by applying pressure around each of the rings made by the wells. Replace the plate's plastic lid snuggly over the plate and gas-permeable film (Fig. 2.1). Incubate the plates for 18 h in a plant growth chamber, under the same conditions as the seedlings were originally germinated, except on an orbital plate shaker set to 220 rpm.

Maintenance of bacterial colonization

To rinse all floats (plants on mesh), add 1 mL of sterile water to wells of a new 24-well plate. Remove gas-permeable film. Using sterile forceps, transfer floats to wells with water (Fig.

2.1). Rinse by resting for 10 min at room temperature (RT) without agitation. To determine bacterial colonization efficiency of roots rather than their ability to maintain colonization over time, plants can be sacrificed at this step by taking them directly to step 5.1. Fill the wells of a new 24-well plate with 1 mL of plant growth medium. Transfer one mesh to each well. Cover with a gas-permeable seal and incubate for 72 h on the orbital plate shaker at 220 rpm in plant growth chamber (Fig. 2.1). Repeat the rinsing as performed in step 4.1 with floats after the 72 h incubation period.

Collection of bacteria for viable cell counts

The number of bacteria per seedling root can be determined at any incubation timepoint. Colonization can be monitored between 0 h and 18 h, while maintenance can be monitored from 18 h onwards. Plants destined for imaging can proceed directly to section 6.

Remove the seedlings from the mesh (Fig. 2.1). Gently place flame-sterilized forceps below the leaves (but on the leaf side of the mesh), and lightly pinch the stem. Wiggle the seedlings up and away from the mesh to dislodge the root without breaking it. If the root breaks, gently scrape the mesh bottom to collect the full length.

Remove bacteria from plant roots. Transfer the bacteria to wells of a 24-well plate containing 1 mL of ddH_20. Sonicate the samples as described in step 1.3. Using a microscope, look for any remaining bacteria on the root surface on a sonicated sample. If bacteria remain, increase the total sonication time or intensity until no bacteria remain bound, up to the highest level of sonication that does not affect viable cell counts as determined in section 1. Quantify the bacteria on roots by performing serial 10-fold dilutions of the sonicated samples up to a 10^{-6} dilution in bacterial growth medium. Add 50 µL of each dilution to individual agar

plates and spread with sterile glass beads (or bacterial spreader). Incubate plates at the optimal temperature for bacteria until individual colonies are countable.

Once distinguishable, count the number of each colony morphology (as determined in section 1), and calculate CFU of each bacterial species per seedling. Discard any samples showing contamination, as contamination during colonization or maintenance may affect bacterial presence.

Collection of intact plant roots for microscopy

Using forceps, remove the seedlings from mesh as in section 5. Transfer each plant to microscope slides. Place the tip of the root on the slide and drag away from the tip to set the shoot down flush with the slide, ensuring a straightened root for best imaging. Add a drop of water or sterile plant growth medium to the samples to hydrate interfaces between the coverslips and slides. Place a glass coverslip just above the root crown (uppermost boxed region Fig. 2.1) and below the shoot leaves to avoid slanting of the coverslip (to allow for root crown imaging) and press down gently[17]. If using fluorescent bacteria, image using appropriate excitation/emission filters to differentiate bacteria from each other and the plant root[18].

2.4. Results

The well-characterized PGPB *P. simiae* WCS417r is known to colonize the roots of *A. thaliana* in hydroponic culture. This naturally fluorescent bacterium can easily be visualized using microscopy on the roots of seedlings following colonization (Fig. 2.2). Although it is possible to image the full length of these *A. thaliana* seedlings' (4–6 mm length) roots, doing so for many plants would take a prohibitive amount of time. Because most variation across

timepoints and species of bacteria can be captured by imaging the crown, middle, and tip of the root[14] (indicated by red boxes in Fig. 2.1), these regions were prioritized for imaging rather than imaging full root lengths. In the bright-field images of *P. simiae*-colonized *A. thaliana* roots (Fig. 2.2), it is possible to visualize the outline of the roots and root hairs; however, at 18 h of colonization, it is not possible to clearly differentiate colonized versus non-colonized roots using bright-field images. While *P. simiae* displays autofluorescence, we used a strain also engineered to express a yellow fluorescent protein (YFP)[19] with excitation/emission wavelengths of 490-510/520-550 nm[18]. A magnification of 100x was sufficient to clearly identify individual and small aggregates of *P. simiae* cells on *A. thaliana* roots. As shown in Fig. 2.2, laboratories with access to either high-resolution confocal microscopes or less expensive benchtop microscopes can both visualize the presence and distribution of bacteria along the root.

While informative in terms of spatial distribution, microscopy images are not well-suited for quantification of bacterial cells. We thus collected bacteria from the surface of roots using ultrasonication as previously described and validated[9,20]. Three rounds of 12 s of ultrasonication[21] at an amplitude of 40 were sufficient to disrupt the outer surface of the root seedlings (Fig. 2.5) and remove all bacteria while not impacting the bacterial viability. Sonication was used rather than bead-beating methods[9] to better promote dispersal of bacterial aggregates/biofilms. Quantifying CFU/root after 18 h of colonization and an additional 72 h of maintenance showed that *P. simiae* both colonizes and is maintained on the roots of *A. thaliana* in our hydroponic, floating seedling system (Fig. 2.3). The number of CFU/seedling at either timepoint showed good reproducibility across biological replicates performed on different days (Fig. 2.3). The variation observed is common among root colonization assays[22] and is likely due to minor variations of timing, environmental conditions, or plant root size, even among

seedlings germinated at the same time and selected to be similar in size. We observe an increase in the number of CFU/seedling after 72 h in maintenance medium as compared to the numbers observed at the post-colonization 18 h timepoint (Fig. 2.3). This indicates active growth of the colonized bacteria on the plant root occurred during the maintenance stage.

In addition to the utility of this hydroponic assay to quantify individual bacterial colonization and maintenance, it is also applicable to monitoring the association of multiple species on plant roots. To demonstrate this, three species of bacteria isolated from *A. thaliana* grown in natural soil under laboratory conditions were chosen[20]. The isolates were strains of *Arthrobacter nicotinovorans*, *Microbacterium oleivorans*, and *Curtobacterium oceanosedimentum*[23]. This simplified community was chosen due to these species' ability to coexist in liquid bacterial growth media in shaking culture (unpublished data). In addition, these three species can be clearly differentiated on media containing X-gal due to differences in colony morphology and color (Fig. 2.4A). The X-gal does not affect relative growth of any of these bacterial species (unpublished data). These differences in morphology and colony appearance on X-gal allowed us to count the CFU/seedling of each species without antibiotic selection, even in multi-species coculture.

A. nicotinovorans, *M. oleivorans*, and *C. oceanosedimentum* were all colonized and maintained on the root, whether alone or in bacterial coculture (Fig. 2.4B). Each species showed trends that were similar across different biological and technical replicates, even within mixed communities. This demonstrates that the protocol can be used to measure both relative or total CFU/root of each species. Interestingly, when grown alone, no individual species showed an appreciable increase in abundance during the maintenance stage, but the overall CFU/root of the

combined community increased in cocultures, indicating that these bacteria do not prohibit the colonization of the other strains.

For all experiments, plants grown in liquid media without the addition of bacteria as negative controls were always included. No bacteria were visible on these control roots during microscopy (Fig. 2.2), nor were any bacteria detected via plating for CFU. This indicates that sterilization of seeds and using the sterile techniques during this assay were sufficient to keep plants axenic unless purposely colonized.

2.5. Discussion

Plants in all environments interact with thousands to millions of different bacteria and fungi[5,7]. These interactions can either negatively and positively impact plant health, with potential effects on crop yield and food production. Recent work also suggests that variable colonization of crops by PGPB may account for unpredictable plant size and crop yield in field trials[22]. Understanding the mechanisms behind these interactions might allow us to directly manipulate plant-associated microbial communities to aid in healthy plant development, even under stress[24].

Because bacterial colonization of roots and their maintenance in the rhizosphere is critical for plant-microbe interactions[9], we wanted to build a system to reproducibly visualize and quantify these bacterial behaviors. This hydroponic, floating seedling growth system allows for microscopic imaging and quantification of bacterial populations on the roots of *A. thaliana*. The described plant-microbe interaction assay integrates beneficial elements of existing experimental protocols. The floating mesh method was based on that from Haney *et al.*[13], which measured initial colonization of *P. simiae* WCS417r on static, floating *A. thaliana* seedlings. In

evaluating this system, strong colonization of *A. thaliana* roots by *P. simiae* was validated, even though a different growth medium from Haney *et al.* was utilized and included orbital shaking during colonization. The inclusion of orbital shaking during both colonization and maintenance facilitates bacterial interactions that might not occur in static culture, as well as reduce anoxic conditions that can inhibit both bacterial growth and plant root health[13]. We also integrated aspects of microbiology-focused protocols designed to support plant root colonization by various bacterial species[8,15,25]. This included a crucial transfer step out of the medium optimized for bacterial colonization and into a medium optimized for plant growth. This transfer to fresh medium also will allow the mechanisms underlying bacterial maintenance on roots to begin to be addressed, an approach that may provide insights into the erratic maintenance of PGPB in field trials[6].

This assay was optimized for rapid processing of multiple samples to allow for environmental variables and mixed bacterial communities to be assayed within one multi-well biological replicate. While ultrasonication has been previously shown to be sufficient for disruption and collection of rhizosphere bacteria, the 24-well plate and multi-prong horn attachment quickens sample processing. This cell viability calculation approach to quantifying bacterial presence could be complemented by, or expanded to include, qPCR or MiSeq 16S rRNA gene community profiling to determine the relative abundance of more diverse communities of colonizing bacteria[20]. In addition, during imaging, the utility of focusing on just three regions of each plant root to speed the visualization of bacterial presence and localization on the roots is highlighted. The colonization of these different root regions has been shown to differ among bacterial species[14]. Imaging can be performed with either naturally autofluorescent bacteria or genetically tractable bacteria engineered to express a fluorescent protein.

The methodology described here allows for fast and reproducible evaluation of root colonization by PGPB bacteria, but there are limitations to the conclusions that can be drawn from these experiments. For instance, the ability for bacteria to chemotax towards the root is known to be important for many bacterial species' colonization of plant roots, but this process may not be required within this shaking inoculation system. That said, for studies specifically interested in chemotaxis, the colonization step could be performed in static liquid culture or on the surface of a soft agar medium, where bacteria could be plated distant from the plant, requiring them to actively move towards the root. In addition, a relatively rich growth medium during colonization was used to promote bacterial growth and plant attachment; however, these comparatively high nutrient concentrations may prohibit the examination of bacterial utilization of or competition for plant-derived carbon during colonization. Again, depending on the growth requirements of the bacteria being studied, the colonization medium can be varied to best suit the particular research questions of specific researchers.

This system was designed to be easily amenable to different bacterial and plant growth conditions and to the addition of different environmental stressors and timepoints. However, the methods described here are best suited for measuring bacterial interactions with the roots of *A. thaliana* seedlings. Collection has been optimized for this plant, and larger or more sensitive plants may be intractable in the floating multi-well-plate system. Finally, while the bacteria of interest used here colonize the plant root in liquid culture, for other bacteria it may be necessary to inoculate the plant roots by dip-inoculation or plating on a solid agar medium instead[19].

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2.7. Tables and Figures

Table 2.1. Materials List

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Required Materials			
1.5 mL eppendorf tubes	any	N/A	
24-well plates	BD Falcon	1801343	
Aeraseal	Excel Scientific	BE255A2	
Autoclave	any	N/A	
Bacteria of Interest	any	N/A	Stored at -80˚C in 40% glycerol preferred
BactoAgar	BD	2306428; REF 214010	
bleach	any	N/A	
Conviron	any	N/A	Short Day Light-Dark Cycles: 460-600 µmoles/m²/s set at 9/15 hours light/dark at 18/21˚C, with inner power outlet
Dessicator Jar: glass or heavy plastic	any	N/A	
Ethanol	any	N/A	
Flame	any	N/A	
Forceps	any	N/A	
Incubator	any	N/A	At optimal temperature for growth of specified bacteria
Hydrochloric Acid	any	N/A	
Lennox LB Broth	RPI	L24066-1000.0	
Microcentrifuge	any	N/A	
Micropipetters	any	N/A	Volumes 5 µL to 1000 µL
Microscope (preferably fluorescence)	any	N/A	Could be light if best definition not important
MS Salts + MES	RPI	M70300-50.0	
Orbital Plate Shaker	any	N/A	Capable of running at 220 rpm for at least 96 hours
Petri Dishes	any	N/A	50 mL total volume
Reservoirs	any	N/A	
Spectrophotometer	any	N/A	
Standard Hole Punch	any	N/A	Approximately 7mm punch diameter
Sterile water	any	N/A	
Surgical Tape	3M	MMM1538-1	

Teflon Mesh	McMaster-Carr	1100t41	
Ultrasonicator	any	N/A	
Vortex Mixer	any	N/A	
X-gal	GoldBio	x4281c	other vendors available
Suggested Materials			
24 Prong Ultrasonicator attachment	any	N/A	For sonicating multiple samples at once. Can be done individually
Alumaseal II	Excel Scientific	FE124F	
Glass beads	any	N/A	
Multipetter/Repetter	any	N/A	
Sterile 96-well plates	any	N/A	For serial dilutions. Can be replaced by eppendorf tubes
Biological Materials Used			
Arabidopsis thaliana seeds	any	N/A	We recommend Arabidopsis Biological Resource Center for seed stocks
Arthrobacter nicotinovorans			Levy, *et al.* 2018
Curtobacterium oceanosedimentum			Levy, *et al.* 2018
Microbacterium oleivorans			Levy, *et al.* 2018
Pseudomonas simiae WCS417r			Published in a similar system in Haney, *et al.* 2015. Strain used developed in Cole, *et al. 2017*

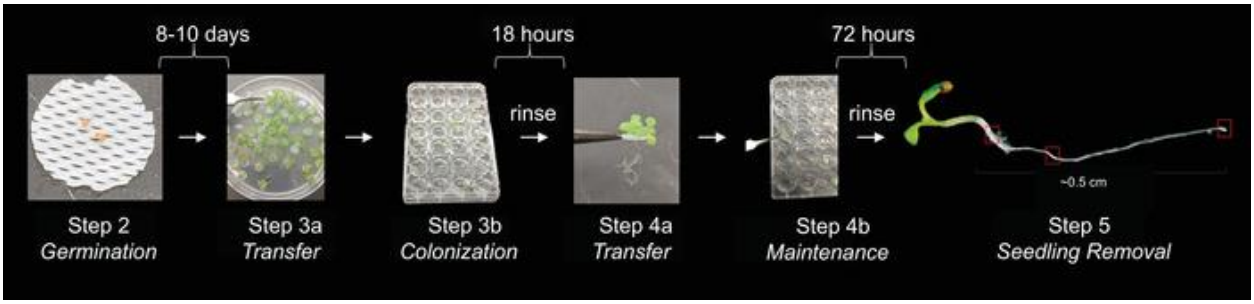

Figure 2.1. Assay for bacterial colonization and maintenance on *A. thaliana* roots.

A. thaliana seedlings grown on sterilized plastic mesh were transferred to a growth medium optimized for bacteria [here, 0.1 x LB (Luria Broth) Lennox]. Bacteria then colonized the root over 18 h while the plant floated in shaking liquid. Following a rinse, the colonized float was transferred to a growth medium optimized for plants (0.5x MS + MES) for 72 h to test for maintenance of bacteria on the roots. The float was then rinsed, and the plant with any attached microbes was removed for analysis (quantification of CFU/seedling or imaging by microscopy).

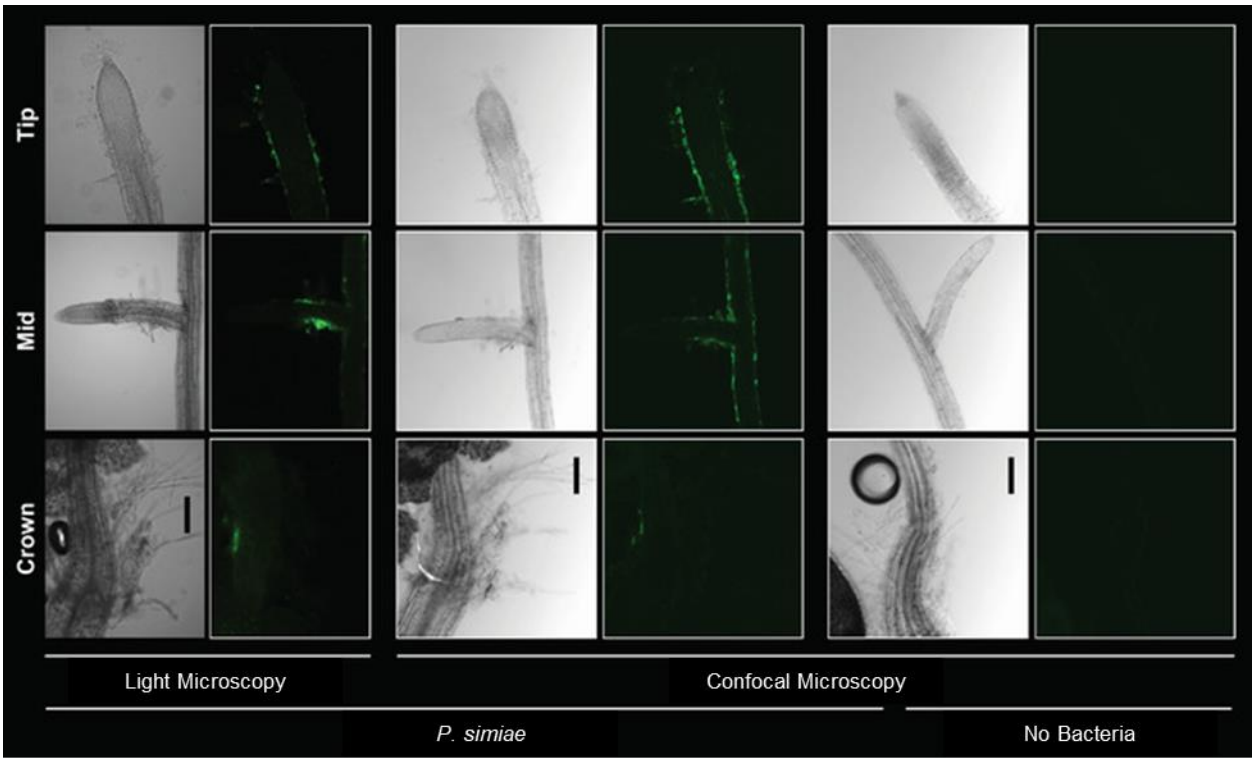

Figure 2.2. Visualizing *P. simiae* colonization of roots with fluorescent microscopy.

P. simiae (false-colored green) colonized *A. thaliana* roots and was maintained on the root following transfer to plant-growth medium. Root crown (left), mid-length (center), and tip (right) at 40x magnification are shown from areas indicated in Figure 2.1. The top two rows show the bright-field and fluorescent images of roots colonized by *P. simiae* (imaged by epifluorescent microscopy). The same roots were also imaged by a confocal microscope (center two rows). The no-bacteria negative control in the two bottom rows showed no colonization. Scale bars represent 50 µm.

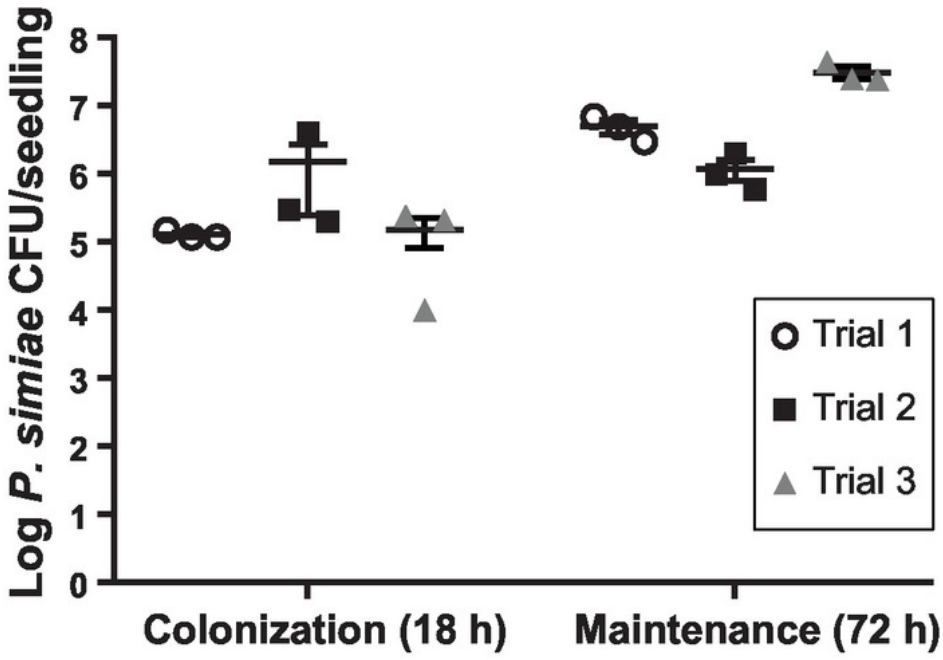

Figure 2.3 Quantification of *P. simiae* on *A. thaliana* roots.

Total number of *P. simiae* viable cells recovered per *A. thaliana* seedling following 18 h of colonization or 72 h of maintenance. Three individual biological replicates are shown, each containing three technical replicates of two seedlings per float. The numbers shown are the means from the technical replicates, while bars represent standard errors of the means.

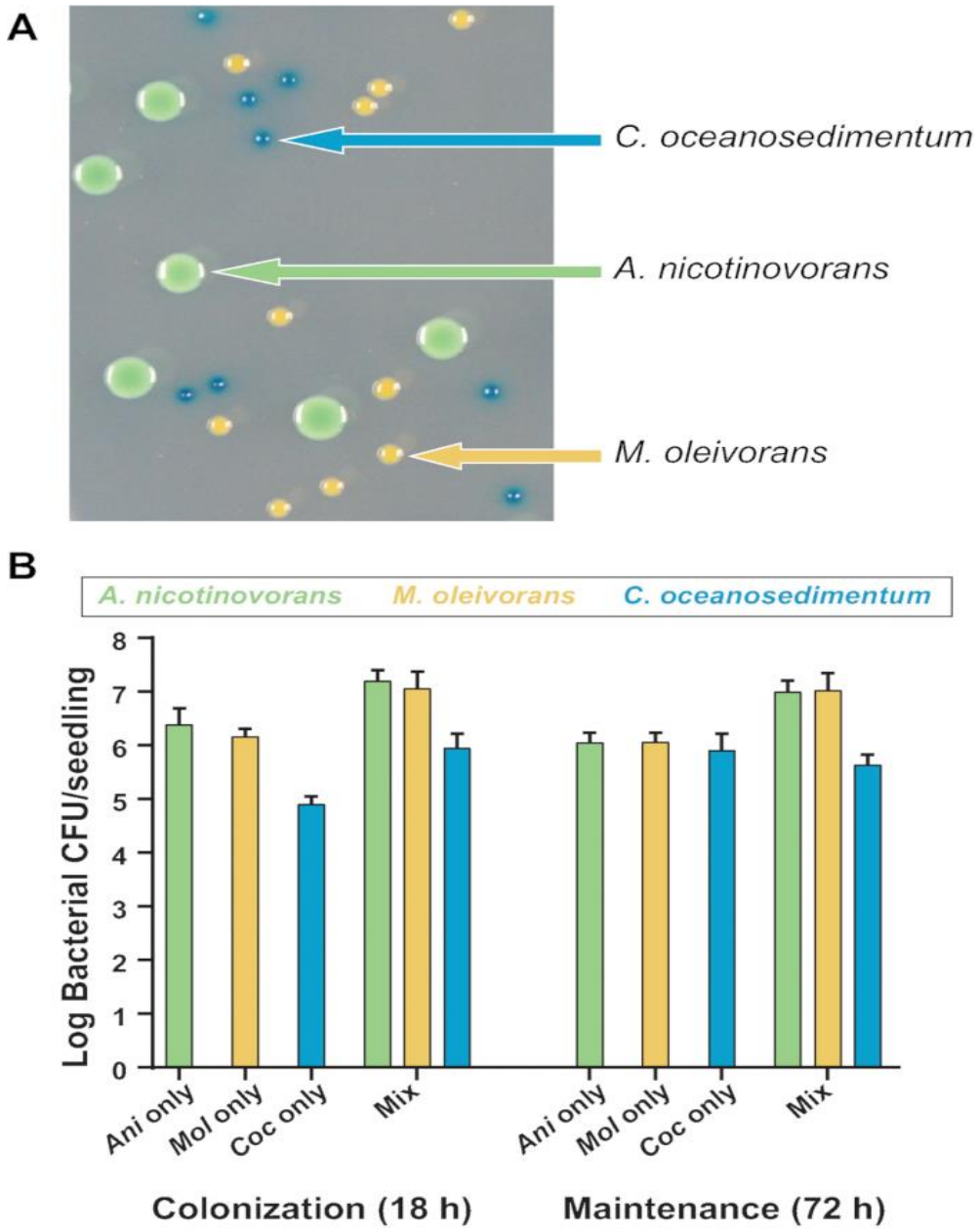

Figure 2.4. Quantification of colonization and maintenance of a mixed bacterial community on *A. thaliana* roots.

(A) Colonies of *A. nicotinovorans*, *M. oleivorans*, and *C. oceanosedimentum* can be differentiated on X-gal-containing agar medium by colony morphology and color. (B) Roots of 10 day-old seedlings were inoculated with approximately $3x10^5$ CFU/mL of each of the three

strains. Shown are total CFU/seedling recovered of each species following 18 h of colonization or 72 h of maintenance when colonized either alone or in a three-member bacterial community. Two biological replicates, each comprising two technical replicates of two seedlings per float, are shown. The numbers shown are the means from the two technical replicates, while bars represent standard errors of the means.

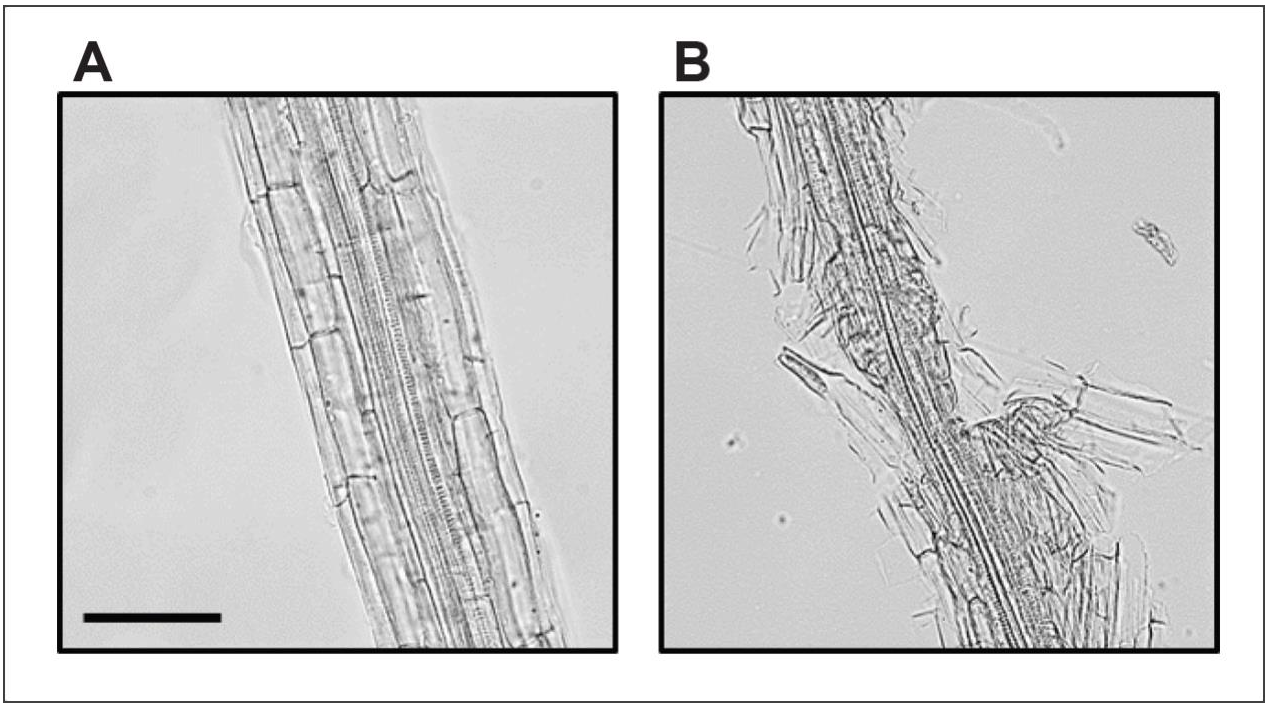

Figure 2.5. Ultrasonication disrupts the root surface.

To dislodge bacteria from the surface of the root, whole plants were sonicated, and the bacteria was released into the liquid, which was serially diluted and plated for quantification of CFU/seedling. (A) An intact seedling is (B) structurally disrupted following ultrasonication.

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CHAPTER 3: BACTERIAL COMMUNITY MEMBERS INCREASE BACILLUS SUBTILIS MAINTENANCE ON THE ROOTS OF ARABIDOPSIS THALIANA

3.1. Overview

Plant growth-promoting bacteria (PGPB) are used to improve plant health and promote crop production. However, since some PGPB (including *Bacillus subtilis*) do not maintain substantial colonization on plant roots over time, it is unclear how effective PGPB are throughout the plant growing cycle**.** A better understanding of the dynamics of plant root community assembly is needed to develop and harness the potential of PGPB. While *B. subtilis* is often a member of the root microbiome, it does not efficiently mono-associate with plant roots. We hypothesized that *B. subtilis* may require other primary colonizers to efficiently associate with plant roots. We utilized a previously designed hydroponic system to add bacteria to *Arabidopsis thaliana* roots and monitor their attachment over time. We inoculated seedlings with *B. subtilis* and individual bacterial isolates from the native *A. thaliana* root microbiome either alone or together. We then measured how the co-inocula affected *B. subtilis*' ability to colonize and maintain on *A. thaliana* roots. We screened 96 fully genome-sequenced strains and identified five bacterial strains able to significantly improve the maintenance of *B. subtilis*; we then characterized three of these strains for effects on *B.* subtilis colonization and detachment kinetics and spatial localization on the root. These rhizobacteria also increased the maintenance of two strains of *Bacillus amyloliquefaciens* commonly used in commercially available bioadditives. These results not only illustrate the utility of this model system to address questions about plant-microbe interactions and how other bacteria impact the ability of PGPB to maintain their

relationships with plant roots, but also may help inform future agricultural interventions to increase crop yields.

3.2. Introduction

As criticism around environmentally costly chemical fertilizers and pesticides increases [1,2], scientists are focusing on developing microbe-based agricultural treatments[3,4]. Bacterial species that increase plant growth or crop yield, regardless of mechanism[5–8], have been defined as Plant Growth-Promoting Bacteria (PGPB)[9]. In laboratory settings, these bacteria colonize what is collectively known as the rhizosphere (including the root surfaces and the areas directly surrounding plant roots)[10,11]. Still, some studies indicate PGPB may not reproducibly maintain their root associations over extended periods of time, in spite of indications that such maintenance may be required for consistent beneficial effects on plants[12,13].

Several species of *Bacillus* are currently used as agricultural PGPB[1,14–17], in part due to their ability to form hardy spores, which allow commercial formulations to remain shelf-stable for over a year[18]. However, as with other PGPB, one concern surrounding the use of *Bacillus* PGPB is that they may not attach to the root or maintain their colonization to levels needed to elicit beneficial effects on the plant[12]. Indeed, since *B. subtilis*' beneficial effects on plants appear to rely on biofilm formation[19], prolonged plant-root interactions may be required for desired PGPB effects. Thus, developing mechanisms to explicitly enhance the maintenance of PGPB on plant roots over time may enable us to improve the beneficial impacts of microbes on crops. Previous work has indicated that *B. subtilis* biofilm formation can be influenced by interactions with neighboring microbes[20,21]. Documented examples of biofilm synergy (i.e. mixes of bacteria increasing biofilm biomass beyond simply additive effects)[22] led us to consider the

possibility that *B. subtilis* and other *Bacillus* PGPB might form more stable associations with plant roots when in the presence of neighboring plant-derived microbes.

Here, we test the hypothesis that native rhizosphere bacteria can promote maintenance of *B. subtilis* on *A. thaliana* roots. We chose *A. thaliana* as our plant host because similar studies using *Bacillus* PGPB have been conducted with this model plant[23]. To test this hypothesis, we used our previously developed hydroponic plant-growth system[24] to screen a library of rhizosphere-derived bacterial isolates to find strains that increase *B. subtilis* maintenance on *A. thaliana* seedling roots. We identified three bacterial isolates that reproducibly increased *B. subtilis* colonization and maintenance on *A. thaliana* roots. In addition, co-culture with each of these three strains either alone or in combination increased maintenance of agriculturally relevant species of *Bacillus.* Taken together, these data suggest that mixed bacterial interactions can prolong colonization of *Bacillus* on plant roots and that application of multispecies inoculants might further improve PGPB-based agricultural interventions [25].

3.3. Materials and Methods

Bacterial strains and growth conditions

All *Bacillus* and rhizosphere isolates used in this paper are listed in Supplemental Table 1. Overnight cultures of *B. subtilis* ES748 and ES749 were grown on Lysogeny Broth (LB)-Lennox medium (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl per liter) at 30 °C. For inoculation, bacteria were suspended in 10 mM $MgCl_2$ + 15% glycerol to a concentration of ~$5x10^7$ CFU per mL. Selection for growth of *Bacillus* strains following collection of CFU from mixed bacterial samples, bacterial suspensions were grown on agar plates containing 80%

Mannitol Salt Agar Medium and 20% LB-Lennox medium. When needed, chloramphenicol and/or erythromycin-lincomycin (MLS) were used at 5 μg/ml and 1 μg/ml, respectively. Ninety-six bacterial strains were previously isolated from the roots of *A. thaliana* grown in natural soil[26,27]. Bacterial strains were made as described above into 100 μL aliquots. Aliquots were thawed at room temperature prior to inoculation.

Colonization of A. thaliana Roots in a Hydroponic Growth System

A. thaliana ecotype Col-0 seeds were grown hydroponically, essentially as described[24]. Sterilized mesh disks (Stretchable High-Temperature PTFE Plastic Mesh 0.045" x 0.025" opening, 18" Wide Mc-Master-Carr 1100T43) of 0.5 cm diameter were cut using a standard hole punch, sterilized by autoclaving, and distributed in a single layer across the surface of agar medium plates containing 0.5x Murashige-Skoog (MS) salts with 50 mg/L MES buffer (Ethylenediaminetetraacetic acid ferric sodium (NaFe-EDTA) constituting 5 ml/l of a stock solution containing 5.57 g $FeSO_4$. $7H_2O$ and 7.45 g Na_2-EDTA). Two surface-sterilized seeds of *A. thaliana* previously stratified at 4 °C were placed on each mesh disk and the entire plate was sealed with gas-permeable tape (BS-25 Aeraseal Excel Scientific) before being placed in a Conviron incubator set for long-day plant growth (16 hours of light, 21 °C daytime, 18 °C at night). Seeds were germinated between 8-12 days.

A. thaliana Col-0 seedlings were colonized with bacterial isolates as previously described[24] with some modifications. Germinated seedlings embedded in sterilized mesh were transferred to 24-well plates containing 0.1x LB liquid medium. 10 μL of each bacterial suspension of ~$5x10^7$ CFU per mL were added to the liquid medium. After covering with a gas-permeable membrane and replacing the lid, plates were incubated in a Conviron incubator set for

short-day plant growth (9 hours of light, 21 °C day, 18 °C night). In the incubator, the plates were placed on a shaker set at 200 rpm to prevent anoxia of the medium [24,28].

Maintenance of Colonization of A. thaliana Roots in a Hydroponic Growth System

Following 20 hours of incubation, mesh disks containing seedlings were removed from the wells and transferred to wells of a 24-well plate containing 1 ml of 10 mM $MgCl_2$ and allowed to stand at room temperature for 10 minutes to remove bacteria not tightly associated with the roots. At this point, seedlings were either used for *A. thaliana* hydroponic maintenance assays or collected for quantification of CFU from *A. thaliana* seedlings. To assay hydroponic maintenance, after the 10 min 'rinse', mesh with seedlings were transferred to a 24-well plate filled with 1.0 mL of 0.5x MS liquid media. After covering with a gas-permeable Aeroseal and the 24-well plate lid, the 24-well plate was transferred to an orbital shaker set to 220 rpm in a Conviron incubator set for short-day plant growth.

Quantification of Bacterial CFU from *A. thaliana* Seedlings

Following either *A. thaliana* hydroponic colonization or maintenance, mesh disks and their embedded seedlings were removed from wells and transferred to wells of a 24-well plate containing 1 ml of 10 mM $MgCl_2$ and allowed to stand at room temperature for 10 minutes to remove bacteria not tightly associated with the roots. Both seedlings were removed from their mesh using flame-sterilized forceps and transferred to wells of a 24-well plate containing 1 mL of 10 mM $MgCl_2$. Each plate was sealed with a gas impermeable Alumaseal and sonicated (Qsonica Sonicator q700) three times with 20 second pauses in between each round using the following program: amplitude 40, 12 second process time, 2 second pulse on, 1 second pulse off.

It was empirically determined that Mannitol-LB agar plates enable *B. subtilis* and *B. amyloliquifaciens* to grow while minimizing or eliminating the growth of the other 22 co-cultured strains. 10-fold serial dilutions in 10 mM $MgCl_2$ were plated on 0.1x LB with and without 6 µg/ml Erythromycin + 5 µg/ml Chloramphenicol antibiotics to monitor overall bacterial growth across wells, as well as on Mannitol-LB agar plates with or without 6 µg/ml Erythromycin + 5 µg/ml Chloramphenicol antibiotics to select for *Bacillus* strains.

Serial dilutions were plated on half-strength LB and full-strength LB with 6 µg/ml Erythromycin + 5 µg/ml Chloramphenicol and were counted after 1-2 days of incubation at room temperature. Centrifuged samples plated on Mannitol-LB plates were counted after 3-5 days later. All results represent at least three replicates performed on separate days, each of which contained at least three seedling-floats per sample type.

Imaging of Bacteria on *A. thaliana* Seedlings

For imaging, colonization and maintenance of *A. thaliana* were performed as described above in the "Colonization" and "Maintenance" steps but using a *B. subtilis* strain containing a constitutive transcriptional mYPet reporter. Regions of the roots were selected for imaging based on their approximate locations relative to either end of the seedling to avoid biasing of collected images. Root attachment images were taken with a Zeiss-710 laser scanning microscope (LSM) (Zeiss, Oberkochen, Germany) and were processed and linearly adjusted using ImageJ[29]. All results represent at least 3 biological replicates performed on separate days, each containing at least three technical replicates.

Phylogenetic Trees

To build the phylogenetic trees, we followed the protocol established by Finkel et al.[30]. Briefly, we used a supermatrix approach to build the phylogenetic tree of the SynCom isolates, as described by Levy and colleagues[31]. Briefly, we scanned 120 previously defined marker genes across the 185 rhizosphere isolate collection genomes using the hmmsearch tool from the hmmer version 3.1b2[32]. We selected 47 markers that were present as single copy genes in 100% of our isolates and aligned each individual marker using MAFFT[33]. Low quality columns in the alignment were filtered using trimAl[34]. Afterward, all filtered alignments were concatenated into a superalignment. Phylogeny was inferred using FastTree version 2.1[35] via the WAG model of evolution.

Statistical analysis

Calculation of variance (one-way ANOVA) and mean comparison between treatments was carried out based on the Dunnett's multiple comparison test at the 0.05 probability level using Graphpad PRISM version 8.3.1 for Windows, GraphPad Software, (La Jolla California USA).

3.4. Results

Monitoring loss of *B. subtilis* root colonization in a hydroponic system

To begin investigating the kinetics of *B. subtilis* colonization and maintenance, we utilized a hydroponic growth system (Figure 3.1) previously published by our laboratory[24]. Briefly, we germinated sterile *A. thaliana* seedlings on agar on top of small pieces of mesh; when germinated in this manner, the roots migrated to the opposite side of the mesh from the

cotyledons. The mesh allowed the seedlings to be easily transferred to liquid wells containing various bacterial strains. After 20 hours of inoculation to permit bacteria to colonize the roots, we gently washed and transferred the plants and their mesh to 0.5x MS, a plant minimal salts medium. Following transfer to 0.5x MS, we removed and rinsed the plants (at 0, 12, 24, 48, 72, and 96 hours post transfer) for collection; we removed bacteria from the surface of the roots through sonication and measured the relative number of *B. subtilis* CFU. Within our system, *B. subtilis* NCIB3610 (hereafter simply *B. subtilis*) repeatedly colonized *A. thaliana* seedlings to ~1 x 10^4 CFU/seedling (Fig 2A). However, following transfer to the plant minimal salts liquid medium, *B. subtilis* CFU declined sharply the first 24 hours, at which point the titer leveled out at approximately two log-fold below the initial colonization levels. We therefore set out to determine whether bacterial isolates from the native rhizosphere of *A. thaliana* could individually colonize and maintain on roots or were able to impact *B. subtilis*' association with plant roots.

Maintenance of root colonization by native rhizosphere isolates

During the design of our hydroponic bacterial-plant-root colonization and maintenance system (Figure 3.1), we discovered that some bacteria were better able to colonize and maintain on roots than others[24]. We therefore wanted to more broadly identify which members of a natural plant microbiome were able to maintain an association with root seedlings over time. We aimed to not only identify individual strains as potential PGPB, but also to look for phylogenetic patterns of the bacteria and their behaviors to determine whether particular bacterial clades were more consistently able to associate with plant roots.

To begin, we screened 96 fully genome-sequenced bacterial strains originally isolated from the rhizosphere of *A. thaliana* ecotype Col-0[26,27] for isolates that were able to maintain their colonization over 24 hours. We elected to set the cutoff for "maintenance" as being at least one log-fold higher than the number of *B. subtilis* in the same experimental replicate after 24 hours of maintenance. Our initial screen identified 51 of these 96 bacterial isolates that were able to maintain on the root at least one log-fold higher titer than *B. subtilis* (Figure 3.2B). Thus, 61% of the tested isolates were classified as "Maintainers" and 39% (including *B. subtilis*) were classified as "Non-Maintainers." These designations did not show any obvious correlation to the phylogeny of the bacteria (see Figure 3.2B and Figure 3.6).

Identification of strains from the rhizosphere that promote *B. subtilis* maintenance on roots

We were intrigued that not all the native *A. thaliana* rhizosphere isolates were able to maintain their associations with roots over time in monoculture, despite being originally isolated from the rhizosphere of the same *A. thaliana* ecotype. Based on existing literature that indicates bacterial interactions can increase biofilm biomass[22], we therefore wondered whether the presence of a Maintainer could increase persistence of a Non-Maintainer such as *B. subtilis*. Using a strain of *B. subtilis* engineered to express a constitutive fluorescent protein that is also resistant to multiple antibiotics (ES749 *B. subtilis* NCIB3610 *amyE*::P$_{spacC}$-*mTurq* (*cm*^{R}); *lacA*::P$_{tapA}$-*mYpet* (*erm*^{R}))[36], we co-inoculated each of the 96 rhizosphere isolates with *B. subtilis* at similar initial CFU/mL on the *A. thaliana* seedlings. Following one day of incubation in 0.5x MS, we compared *B. subtilis* CFU per seedling when inoculated alone or with another bacterium. Using this method, we flagged 22 of the 96 strains as putative helper bacteria: in coculture, these 22 strains appeared to increase the CFU of *B. subtilis* maintenance by at least a log-fold relative

to when *B. subtilis* was inoculated alone (Figure 3.3). Not all strains were initially identified as Maintainers on their own (Figure 3.2B), suggesting that the ability of individual strains to "help" *B. subtilis* maintain may not be dependent on their own ability to persist on roots. It is important to note that, for technical reasons, we did not quantify the CFU of these strains but only examined their effects on *B. subtilis.*

To validate and confirm which of these 22 strains could reliably increase maintenance of *B. subtilis* on the roots of *A. thaliana,* we quantified the effect of these strains in a secondary screen across multiple days and with additional replicates relative to our initial screen (at least three mesh floats containing two seedlings grown in individual wells/sample type/day) (Figure 3.3). Values above the line of neutrality (middle "1" line on the y-axis) indicated co-culturing had a positive effect on *B. subtilis* adherence. Co-culture with five strains (*Agrobacterium* sp. ES981, *Variovorax* sp. ES1063, *Methylobacterium* sp. ES1072, *Methylobacterium* sp. ES1084, and *Brevundimonas* sp. ES1115) significantly increased *B. subtilis* maintenance ($p<0.0001$ for ES981, ES1063, ES1072, and ES1084; $p<0.05$ for ES1115). While two *Methylobacterium* are within this group, the overall phylogenetic diversity of these helper strains is broad and appears un-conserved across phylogeny. The other 17 strains that passed our initial screen but not our more rigorous secondary screen were likely artifacts from high levels of biological variability for these strains. Of the five strains, we chose three to focus on during subsequent experiments (*Agrobacterium* sp. ES981, *Variovorax* sp. ES1063, and *Methylobacterium* sp. ES1084) due to their lower sample variance compared to ES1072 and stronger significance compared to ES1115 (Figure 3.3). These strains were also found to be Maintainers when in monoculture association with the root (Figure 3.2B).

Effects of co-colonization on the spatial distribution of *B. subtilis* on the root surface

Bacillus species show preferences in the location of their attachment along the length of plant roots, presumable based on the zone of root cell differentiation[37]; furthermore, the colonization preferences of some *Bacillus* have been shown to be affected by the presence of other bacteria[38]. Finally, bacterial biofilms found on natural surfaces often comprise multiple species[39]. Thus, we wondered whether and how these three helper strains were affecting the spatial localization patterns of *B. subtilis* on *A. thaliana* roots. We sought to determine whether different bacterial species were in close contact on the roots or if they were spatially segregated.

To address this question, we performed the maintenance assay as before, but now also collecting seedlings following the colonization step and prior to the transfer to 0.5x MS. In addition, we used a *B. subtilis* strain engineered to constitutively express the mYPet fluorescent protein (ES748 *B. subtilis* NCIB3610 *lacA*::P_{tapA}-*mYPet*, erm^R)[36]. Other than the fluorescent gene construct (*mYPet* vs. *mTurq*) and lack of the chloramphenicol resistance gene, this strain was genotypically identical to the parental *B. subtilis* strain used in our original screening assays; however, its brighter fluorescence allowed us to better visualize the bacteria along the root.

Using confocal laser fluorescence microscopy, we imaged the roots at four sections along their length during colonization and maintenance: the crown (where the root meets the shoot), the upper middle (in the half closest to the crown) and lower middle (in the half closest to the root tip) sections of the main root, and the tip of the main root. We saw the most consistent differences across samples at the lower-middle-section of the root (shown in Figure 3.4A; see Figure 3.7 for representative images at all four locations along the root length). Following colonization, only a few *B. subtilis* cells were visible along the surface of the root; in comparison, slightly more fluorescent bacteria were found when *B. subtilis* was co-inoculated

with the *Methylobacterium* sp, and clumps of fluorescent bacteria were visible when *B. subtilis* was co-inoculated with either the *Agrobacterium* sp. or the *Variovorax* sp.

Following maintenance, we also observed differences in *B. subtilis* abundance when in association with *A. thaliana* alone or with co-colonizing bacteria: it was rare to visualize any fluorescent cells on roots inoculated with *B. subtilis* alone; in contrast, fluorescent *B. subtilis* cells were always apparent when *B. subtilis* was co-inoculated with either the *Agrobacterium* sp. or the *Variovorax* sp. There appeared to be fewer *B. subtilis* cells at the lower-mid-root location following colonization with the *Variovorax* sp. as compared to co-inoculation with the other two Allies; however, *B. subtilis* cells were more prevalent following maintenance when co-inoculated with the *Variovorax* sp. compared to when co-inoculated with either the *Agrobacterium* sp. or the *Methylobacterium* sp.

Because none of these three helper strains were engineered to express a fluorescent protein and did not show appreciable autofluorescence, we could not determine their exact locations on the root. However, based on DIC images, it appeared that these species were often in direct contact with the *B. subtilis* cells. For example, in co-colonization with the *Agrobacterium* sp., it appears that small clumps of *B. subtilis* cells are encased in a larger colony of nonfluorescent cells, which appear to be *Agrobacterium* cells. This apparent mixing is more obvious when visualized through 3D reconstruction of confocal microscopy images taken at multiple Z-planes.

The most promising strains increase colonization and maintenance of *B. subtilis*

To determine how these three strains affected the kinetics and dynamics of *B. subtilis*' association with the plant root, we performed co-inoculation assays as before but now removed

plants for determination of bacterial CFU at multiple timepoints (at the initial transfer after colonization, after one day of maintenance and after three days of maintenance). Co-inoculation with each of the three strains increased *B. subtilis*' colonization by at least one log-fold as compared to *B. subtilis'* colonization alone (Figure 3.4B). Importantly, this increase in colonization was not due to a general increase in *B. subtilis* growth in the co-culture colonization media itself, as *B. subtilis* CFU counts from the liquid medium showed no effect of co-inoculation (Figure 3.8). In addition, while the overall titer of *B. subtilis* CFU/root decreased between one and three days of incubation in maintenance medium, we observed approximately the same trends at both time points. Thus, in future screening efforts to identify isolates able to increase of *B. subtilis* maintenance, one day of maintenance incubation may be sufficient. It is worth noting that our assays require destructive sampling, so we could not compare the colonization and subsequent maintenance on individual plants over time.

Maintenance of agriculturally relevant *Bacillus* PGPB is improved by strains that impact *B. subtilis*, both individually and in combination

We used the undomesticated strain[40] *B. subtilis* NCIB3610 for our screens due to the wealth of information about its behavior[41] and range of available genetic tools, as well as the observation that it inhibits invasion by the pathogenic *Ralstonia solenacearum* on tomato plants[19]. However, we next wanted to determine whether the three strains that increased the maintenance of *B. subtilis* NCIB3610 were also able to promote the maintenance of other *Bacillus* species, including *Bacillus* strains used as PGPB additives in agricultural interventions. We specifically tested whether these strains or their combination could affect the maintenance of *B. amyloliquefaciens* strains GB03 and FZB42, which are widely used as PGPB additives in agriculture[25,42], and had been previously compared with *B. subtilis* 3610 in terms of their biofilm

forming capabilities (albeit in a slightly different growth format[43]). Interestingly, FZB42 has also been shown to lose colonization over time on lettuce plants[18].

Based on the results from our microscopy imaging (Figure 3.4A), it appeared that the three helper strains we had identified affected localization of *B. subtilis* differently. We therefore considered whether each individual strain might affect *B. subtilis*' localization through distinct mechanisms that could be mutually beneficial if combined. This concept is consistent with the idea that multispecies communities can exhibit emerging properties that arise through complex multi-way interactions[44]. We therefore wanted to determine both how each of the three strains individually would affect the maintenance of these *Bacillus* spp., but also whether their combination would have additive or synergistic impacts on the PGPB.

We inoculated plants with each separate *Bacillus* strain and the three helper strains either alone or in a 1-to-1-to-1 mix. After one day of maintenance, we plated the sonicated root samples on selective mannitol/LB agar plates without antibiotics and counted *Bacillus* spp. CFU (note that none of the helper strains grew on mannitol/LB, while all the *Bacillus* strains did). We then compared the number of the *Bacillus'* CFU in the presence of the other strains compared to the *Bacillus*-only inoculation (Figure 3.5).

We found that co-inoculation with each of the three strains individually did increase the maintenance of *B. subtilis* and both *B. amyloliquefaciens* strains (Figure 3.5). However, when all three helper strains were collectively co-inoculated, they did not increase the maintenance of the *Bacillus* strains more than any of the individual helpers did (i.e. there was no synergy of their interactions or additional benefits of combining them) (Figure 3.5). These results indicate that screening for maintenance effects of co-inoculation of *B. subtilis* 3610 with bacterial isolates may be used to identify strains for other species of agriculturally relevant *Bacillus* species.

Additionally, they suggest that in at least some cases, using a combination of strains might not supplement or detract from the effects of the others' presence.

3.5. Discussion

To leverage the potential power of microbe-based agricultural interventions, we must first understand how diverse biotic and abiotic environmental factors mediate plant productivity. This includes studying how mixed microbial species affect phytobiome health and stability. The rhizosphere microbiome of plants grown in natural soils are frequently comprised of hundreds of species[27]. The assembly of these complex plant microbiomes often requires interspecies bacterial interactions, where the presence of certain species facilitates the subsequent growth, maintenance, and succession of other species[45–47]. Indeed, recent studies have found that *Pseudomonas chlororaphis* affects *B. subtilis* NCIB3610 colonization on melon roots[48], while peptidoglycan from *B. cereus* facilitates rhizospheric bacterial commensalism[49]. We therefore reasoned that interbacterial interactions could play a role in the persistence and behavior of *Bacillus* PGPB on plant roots.

B. subtilis is commonly utilized as a PGPB: it has been shown to increase root growth of melon seedlings[50] and inhibit plant pathogen invasion through production of specialized metabolites[19,51]. While *B. subtilis* strains have been included in commercial agricultural bioadditives, other strains of *Bacillus*, such as *B. amyloliquifaciens* FZB42 and GB03, are more widely used for such purposes[12,5]. That said, *Bacillus* species added to plant growth systems as PGPBs do not always maintain their initial colonization on roots over time[12,18].

Using our hydroponic growth assay to measure bacterial presence on plant roots, we identified rhizosphere bacteria that could maintain root colonization on their own as well as those

that could promote maintenance of the model bacterium *B. subtilis* and PGPB strains of *B. amyloliquifaciens* on *A. thaliana*. Despite all tested bacterial co-inoculants being originally isolated from the rhizosphere of *A. thaliana* grown in natural soil [27], only 51 of these 96 bacteria were able to maintain their independent associations with *A. thaliana* roots over time. These results suggest that additional microbe-microbe or microbe-environment interactions may be important to promote the association of these individual rhizosphere bacteria with roots.

In addition, we identified five rhizosphere isolates that significantly ($P < 0.05$) increased the CFU of *B. subtilis* associated with roots (an *Agrobacterium*, *Variovorax*, *Brevundimonas*, and two *Methylobacterium* strains). Thus, overall, a relatively small percentage of the tested isolates can impact these *Bacillus* strains, even though all of the rhizosphere bacteria examined were originally isolated from native soil rhizospheres. These results are consistent with strain specificity being crucial for these interbacterial interactions, an idea supported by the fact that multiple phylogenetically close relatives of the identified helper strains were not able to increase *B. subtilis* maintenance in our assay. When we more deeply explored three of these helper strains (ES981, ES1063, ES1084), we saw that they were able to increase the association of *B. subtilis* with roots over time, while also generally improving *B. amyloliquefaciens*' root associations.

Bacteria belonging to the genera these helpers are a part of (the *Agrobacterium*, *Variovorax*, and *Methylobacterium* clades) have all previously been found in association with plants, but minimal literature explicitly discusses potential interactions of bacteria from these groups with *Bacillus* species. It is known that some strains of *B. subtilis* and *B. amyloliquefaciens* reduce the incidence of disease by pathogenic *Agrobacterium tumafaciens*, which causes crown gall on eudicots, when co-colonized[52]. Notably, although *Agrobacterium* spp. are most commonly considered plant pathogens, some species have been shown to have a

phytostimulatory effect[53]. That said, work clearly remains to understand the specifics of the mechanisms by which these particular helper strains either directly or indirectly impact *Bacillus*' association with plant roots.

It is interesting to note that, although these three helper strains consistently demonstrate strong trends towards increasing the association of *Bacilli* with plant roots, there is significant biological variability observed in the data. This is consistent with published papers demonstrating that inoculation of plants with a *Bacillus* PGPB led to increased variance of plant growth, rather than a consistent increase across all plants[12]. However, this increase in variance was only apparent when the individual data points were examined (and the true data points were not obscured by box-and-whisker plots or bar graphs[54]). While this biological variability meant that our ability to claim statistical significance from these data in some cases failed, we are confident that the trends (that these helpers increase *Bacillus* associations with *A. thaliana* roots) are reproducible.

These quantitative measurements were corroborated by confocal fluorescence microscopy images of fluorescently labelled *B. subtilis* co-inoculated with these three helper strains. Our images and movies clearly show how these different species are often in direct contact within the root-associated biofilms. Future work with both strains being fluorescently labeled either using genetic[38,55] or chemical[56] methods would enable their physical relationships on the root to be more precisely described. These approaches would be particularly beneficial for visualizing mixtures of more than two bacteria: in part because inoculation of *Bacillus* species has previously been shown to modify the root endophytic bacterial diversity[57], it would be interesting to see how *B. subtilis* impacts the localization of the helpers along the root rather than just how the helpers affect *B. subtilis.* In addition, gaining an understanding of whether direct

physical interaction between these bacteria is required for increased PGPB association with roots (or whether diffusion of their metabolites is sufficient) may inform methods for future agricultural interventions.

Previous studies have examined the effects of mixed PGPB consortia on plant growth and microbial composition[58–60], but here were we interested in the (related) question of whether, when co-inoculated, multiple potential helper strains showed additive or synergistic impacts on a single PGPB. Multispecies communities are typically more stable that single strains are, and emergent properties can arise in complex multi-way interactions[44]. However, in this case we found that combining helper strains did not increase the maintenance of the PGPB *Bacilli* over that of the most-effective strain alone. Even so, in more complex natural environments, the benefit of applying multiple strains simultaneously might stabilize their impact across different environments; indeed, adjustments to our assay could be made to directly scan a variety of possible environmental perturbations (nutrient availability, salinity, etc.) to identify suites of helper strains able to benefit PGPB under a range of growth conditions.

Novel technical approaches are needed to study phytobiome systems so we can better manipulate and manage phytobiomes in agricultural ecosystems across rapidly changing conditions around the world[61]. While here we focused on the interactions between *Bacillus* species and *A. thaliana*, our hydroponic assay can enable many different plant and bacterial species to be studied[28] under a variety of environmental conditions. Identifying microbes that affect the associations of PGPBs with plant roots could enhance the efficacy of microbial agricultural interventions as well as improve our understanding of complex interkingdom interactions[25,62]. Our work complements those of ongoing studies elucidating the effects of plant inoculation with mixed-species communities[63–65], especially in the context of soils[30,66].

Integrating findings across these different efforts will be essential for supporting translational research from phytobiome microbiology to agronomy.

3.6. Acknowledgements

We kindly thank Jeffery Dangl (UNC-CH) for providing the library of rhizosphere isolates, Isai Salas González (UNC-CH) for constructing the phylogenetic trees, Michael Fischbach (Stanford University) for providing the *B. amyloliquifaciens* strains, Jamie Winshell and the laboratory of Joseph Kieber (UNC-CH) for providing sterilized *A. thaliana* seeds, Curtis McGehee for assistance with bacterial stock preparation, Tony Perdue and the UNC Biology Department Microscopy Core for use of and support for a Zeiss-710 laser scanning microscope, and Cynthia Darnell for ongoing advising. This work was supported by research funds from the National Institutes of Health (GM112981) and the Department of Energy (DE-SC0013887 and DE-SC0019012) to E.A.S. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

3.7. Tables and Figures

Table 3.1. Natural Rhizosphere Isolate Strains

Strain name (Pseudonym)	Dangl Lab Designation	Genus	Solo Maintainer	Helper Screen	Final Helpers
ES0976	CL021	*Ralstonia*	X	X	
ES0977	CL025	*Bacillus*			
ES0978	CL028	*Arthrobacter*	X		
ES0981	CL041	*Agrobacterium*	X	X	X
ES0982	CL045	*Microbacterium*	X	X	
ES0983	CL052	*Paenibacillus*	X		
ES0984	CL058	*Pseudomonas*	X	X	
ES0985	CL063	*Arthrobacter*	X		
ES0986	CL069	*Acinetobacter*	X		
ES0987	CL071	*Acinetobacter*			
ES0988	CL081	*Bacillus*			
ES0989	CL089	*Microbacterium*	X		
ES0990	CL091	*Paenibacillus*			
ES0994	CL125	*Methylobacterium*		X	
ES0995	CL126	*Methylobacterium*	X		
ES0997	CL129	*Methylobacterium*	X		
ES0999	CL136	*Methylobacterium*			
ES1002	CL143	*Methylobacterium*	X		
ES1005	CL154	*Leifsonia*			
ES1037	MF069	*Bacillus*		X	
ES1038	MF071	*Bacillus*			
ES1039	MF077	*Microbacterium*	X	X	
ES1040	MF079	*Dyella japonicum*	X	X	
ES1042	MF092	*Stenotrophomonas*	X		
ES1043	MF095	*Bacillus*	X		
ES1045	MF103	*Bacillus*			
ES1046	MF105	*Bacillus*			
ES1047	MF106	*Bacillus*		X	
ES1048	MF109	*Leifsonia*	X		
ES1049	MF110	*Variovorax*	X		
ES1050	MF111	*Methylobacterium*			
ES1051	MF112	*Bacillus*	X		
ES1052	MF113	*Pseudomonas*	X		
ES1053	MF114	*Rhodococcus*			
ES1054	MF115A	*Leifsonia*	X		
ES1055	MF123	*Bacillus*			
ES1057	MF131	*Arthrobacter*	X		

ES1058	MF135	*Arthrobacter*	X		
ES1060	MF138	*Luteibacter*	X		
ES1062	MF157	*Leifsonia*	X		
ES1063	MF160	*Variovorax*	X	X	X
ES1064	MF161	*Arthrobacter*	X		
ES1065	MF162	*Arthrobacter*			
ES1066	MF164	*Rhodococcus*	X		
ES1067	MF166A	*Bacillus*	X		
ES1068	MF174	*Methylobacterium*	X		
ES1069	MF177	*Phyllobacterium*			
ES1070	MF178	*Dyella marensis*	X	X	
ES1071	MF181	*Paenibacillus*			
ES1072	MF190	*Methylobacterium*		X	
ES1073	MF196	*Bacillus*			
ES1074	MF212	*Bacillus*	X		
ES1075	MF215	*Bacillus*			
ES1076	MF217	*Paenibacillus*			
ES1077	MF220A	*Sphingomonas*	X	X	
ES1078	MF224	*Agrobacterium*	X		
ES1079	MF231	*Arthrobacter*	X		
ES1080	MF254	*Arthrobacter*	X		
ES1081	MF261	*Leucobacter*			
ES1082	MF267	*Mycobacterium*		X	
ES1083	MF273	*Terracoccus*			
ES1084	MF275	*Methylobacterium*	X	X	X
ES1085	MF278	*Variovorax*	X		
ES1087	MF283	*Mycobacterium*			
ES1088	MF285	*Methylobacterium*			
ES1089	MF292	*Microbacterium*	X		
ES1090	MF295	*Variovorax*	X		
ES1091	MF299	*Streptomyces*			
ES1093	MF302	*Phyllobacterium*			
ES1094	MF303	*Streptomyces*			
ES1095	MF312A	*Chryseobacterium*	X	X	
ES1096	MF314	*Curtobacterium*	X		
ES1097	MF322	*Bacillus*			
ES1098	MF327	*Promicromonospora*	X		
ES1099	MF329	*Luteibacter*	X		
ES1100	MF333	*Luteibacter*	X	X	
ES1101	MF339	*Rhodococcus*	X		
ES1102	MF340	*Cryocola*	X		
ES1104	MF348	*Nocardia*			
ES1105	MF349	*Variovorax*	X		

ES1106	MF350	*Variovorax*	X		
ES1107	MF351	*Streptomyces*			
ES1109	MF360	*Mycobacterium*	X		
ES1110	MF362	*Arthrobacter*	X		
ES1111	MF363	*Rhodococcus*		X	
ES1112	MF365	*Mycobacterium*	X	X	
ES1113	MF366	*Luteibacter*	X		
ES1114	MF370	*Ochrobactrum*	X		
ES1115	MF374	*Brevundimonas*		X	
ES1116	MF375	*Variovorax*	X		
ES1117	MF376	*Burkholderia*			
ES1118	MF384	*Burkholderia*	X		
ES1119	MF395	*Pseudomonas*	X	X	
ES1120	MF397	*Pseudomonas*	X	X	
ES1122	MF491	*Bacillus*			
ES1124	MF499	*Paenibacillus*			

Table 3.2. Laboratory *Bacillus* Strains

Strain name (Pseudonym)	Bacterial species/strain	Genotype	Source (Reference)
ES223	*Bacillus amyloliquifaciens* FZB42	Wild type	Fischbach lab collection
ES414	*Bacillus amyloliquifaciens* GB03	Wild type	Fischbach lab collection
ES748	*Bacillus subtilis* 3610	*lacA*::P_{tapA}-*mYPet*, erm^R	Shank lab collection
ES749	*Bacillus subtilis* 3610	*amyE*::P_{spacC}-*mTurq* (cm^R); *lacA*::P_{tapA}-*mYpet* (erm^R)	Shank lab collection

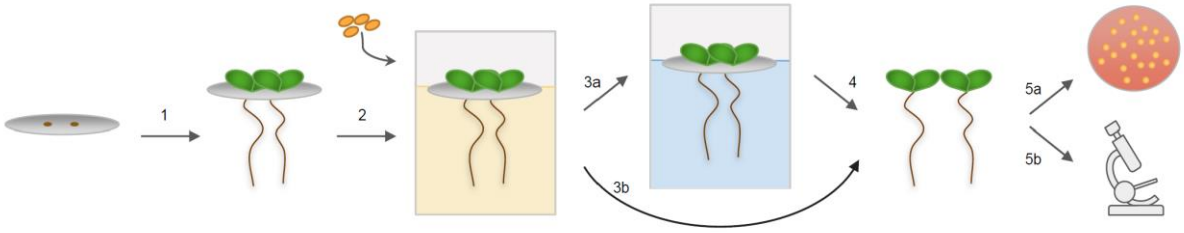

Figure 3.1. Schematic of hydroponic growth system for quantification and imaging of bacterial associations with *A. thaliana* seedling roots.

(1) Two surface-sterilized seeds of *A. thaliana* were germinated on discs of sterile Teflon mesh for 7-to-10 days. (2) Mesh discs embedded with two seedlings were transferred to the colonization liquid medium, into which bacterial strains were inoculated. (3) After 20 hours of colonization, mesh and seedlings were rinsed and either (3a) were transferred to the maintenance medium liquid or (3b) seedlings were removed for immediate sample collection. (4) After 20 hours of colonization, mesh with seedlings were rinsed and seedlings were removed for sample collection, to be either (5a) sonicated in liquid to resuspend bacteria for plating for CFU counting or (5b) transferred to microscope slides for fluorescence microscopy imaging.

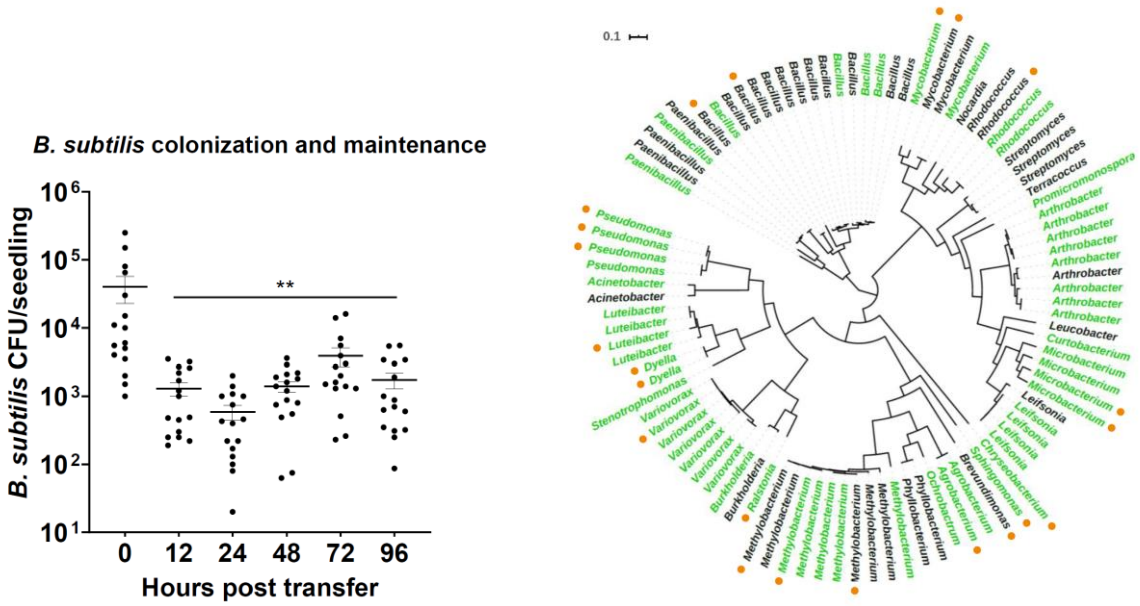

Figure 3.2. Maintenance of initial bacterial colonization.

(A) *B. subtilis* colonizes the hydroponic roots of *A. thaliana* seedlings but does not maintain its association when seedlings are transferred to minimal salt medium for continued incubation. **, $P < 0.01$. Error bars, standard error of the mean. (B) An unrooted phylogenetic tree showing the relationships between the 96 rhizosphere bacterial strains tested for their hydroponic association with *A. thaliana* roots Isolates found to maintain their associations with *A. thaliana* roots at a level at least one log-fold higher than *B. subtilis* are shown in green. The outer ring of orange dots indicates the 22 isolates selected for further study.

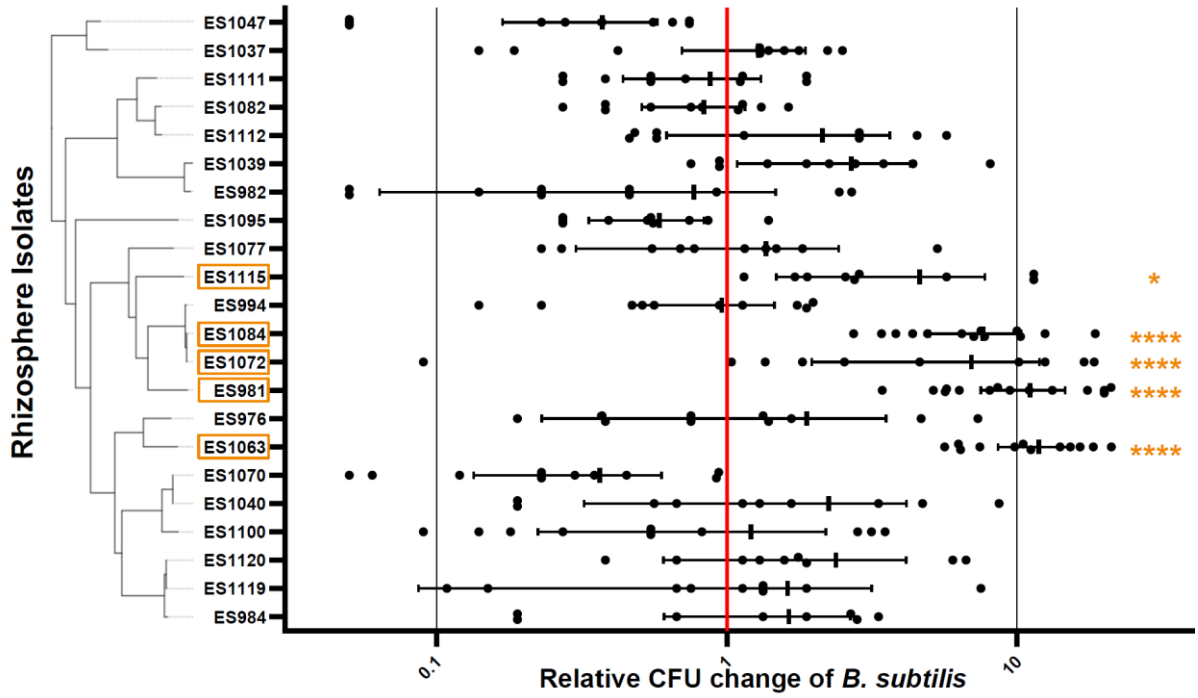

Figure 3.3. Secondary screen of isolates implicated to increase *B. subtilis* maintenance in co-culture.

Of these 22 strains, we identified four strains that reproducibly increased *B. subtilis* maintenance by at least one log-fold (ES981, ES1063, ES1084, ES1115), which are outlined in orange. Differences in *B. subtilis* CFU/seedling when co-inoculated with an isolate are reported as log-fold changes compared to the average *B. subtilis* CFU/seedling in the same biological experiment. Error bars, standard error of the mean. *, $P<0.05$. ****, $P<0.0001$.

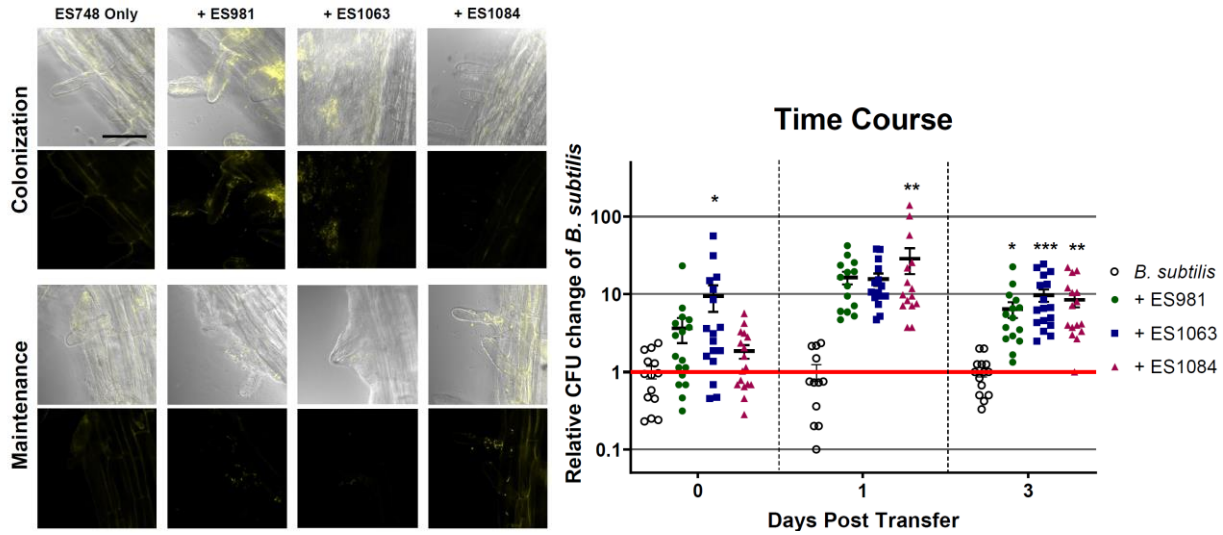

Figure 3.4. Spatiotemporal kinetics of *B. subtilis* association with plant roots in co-culture with rhizosphere isolates.

(A) Distributions of *B. subtilis* on the lower regions of plant roots following colonization (top) and maintenance (bottom) when inoculated either alone or with the indicated strains. Fluorescent cells were false colored yellow in both the DIC-fluorescent image overlays and the fluorescent images, alone. Images from each section were collected from at least two technical replicates of three independent biological replicates of these experiments. Bar, 50 µm. (B) Plant root attachment of *Bacillus subtilis* following colonization (0 days) and maintenance (1 and 3 days) is increased by co-colonization with either ES981, ES1063, or ES1084. Differences in *B. subtilis* CFU/seedling when co-inoculated with another strain are reported as log-fold changes compared to the average *B. subtilis* CFU/seedling of three replicates in the same biological experiment. Error bars, standard error of the mean. *, $P<0.05$. **, $P<0.01$. ***, $P<0.001$.

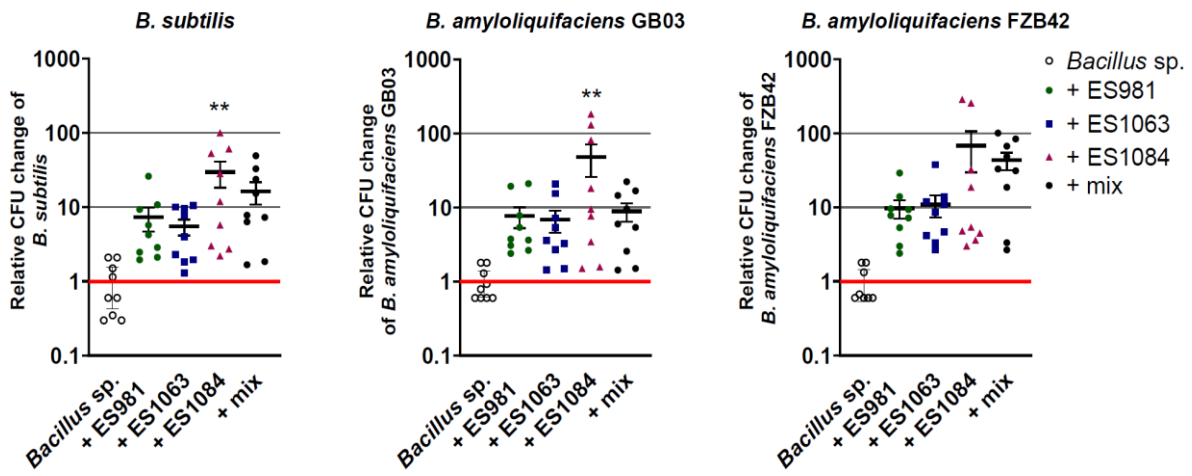

Figure 3.5. Maintenance of PGPB *Bacillus* species' colonization in co-culture with each rhizosphere isolate individually and in combination.

We inoculated three *Bacillus* species, (A) *B. subtilis* ES749, (B) *B. amyloliquefaciens* ES223 and (C) *B. amyloliquefaciens* ES414, either alone or with the addition of ES981, ES1063, and ES1084, either each strain alone or all together ('mix'). Graphs show the differences in *Bacillus* CFU/seedling when co-inoculated with as reported as log-fold changes compared to the average *Bacillus* sp. CFU/seedling of three replicates in the same biological experiment. Error bars, standard error of the mean. *, $P<0.05$. **, $P<0.01$.

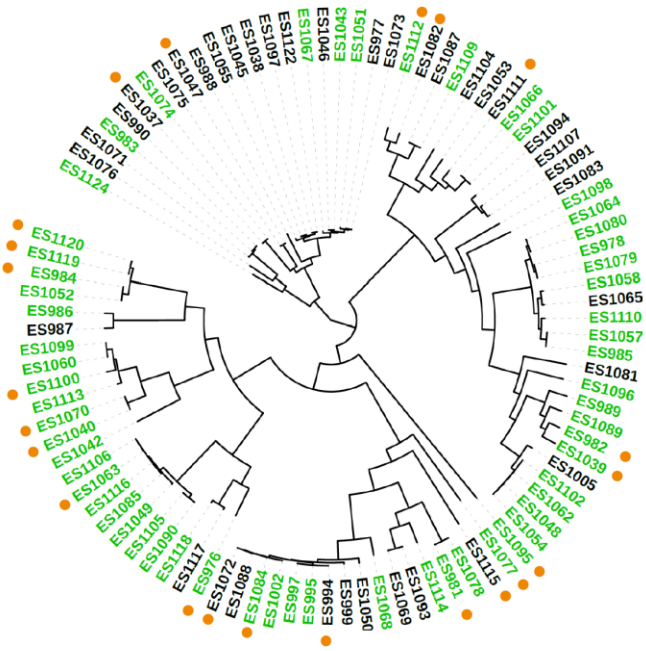

Figure 3.6. Unrooted phylogenetic tree of screened strains based on 47 strain-specific marker genes.

This tree and its coloring is identical to that shown in Figure 3.1, and allows particular isolates of interest to be correlated with ES strain names.

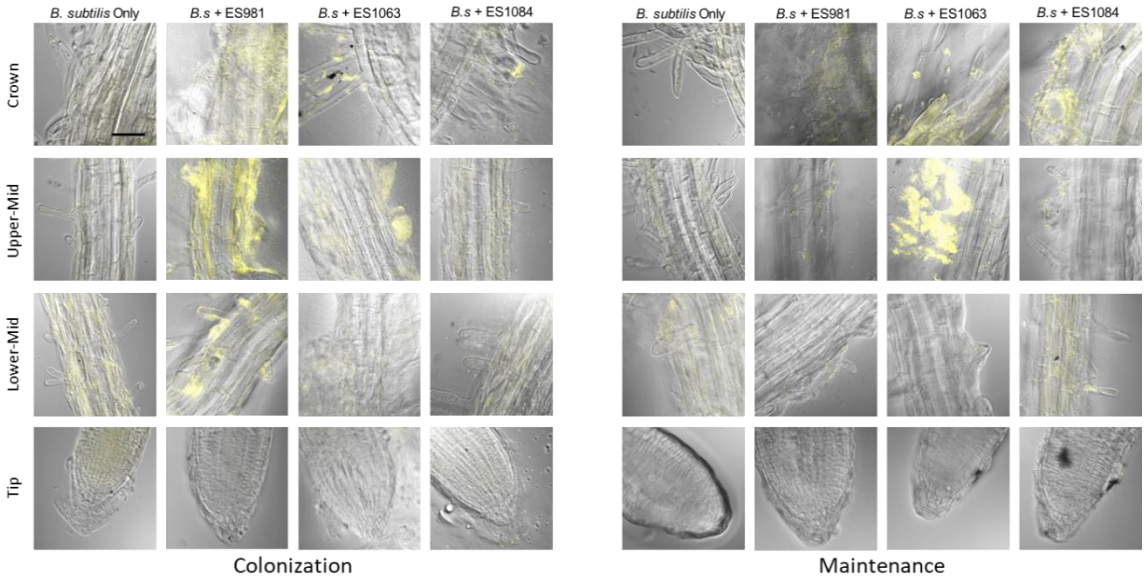

Figure 3.7. Localization of *B. subtilis* following colonization and maintenance either alone or in coculture with the indicated strains.

Images of bacterial associations with the plant roots were obtained by confocal fluorescence microscopy. Panels show overlays of DIC and fluorescent images where the fluorescent cells are false-colored yellow. Bar, 50 µm.

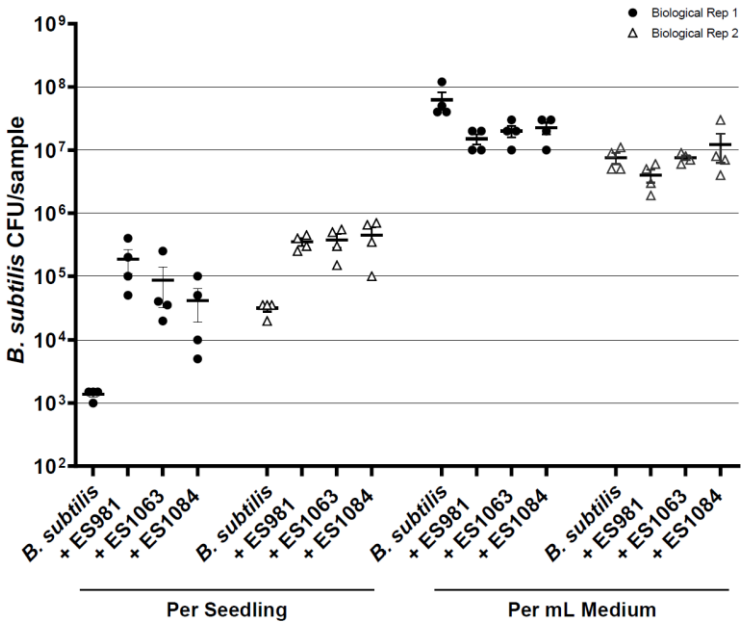

Figure 3.8. CFU of *B. subtilis* on seedling roots and in the liquid growth medium following colonization in mono-inoculation and co-inoculation.

CFU collected from the root fraction are expressed as CFU/seedling, while CFU collected from the liquid medium fraction are expressed as CFU/mL liquid.

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CHAPTER 4: DISCUSSION AND FUTURE EXPERIMENTS

4.1. Summary of Results

My dissertation focuses on defining the kinetics of bacterial-plant associations through novel methods and shows the efficacy of using these methods for studying interactions of Plant Growth-Promoting Bacteria (PGPB) within bacterial communities on the plant root. This work was based on the observation that *Bacillus subtilis*, a PGPB commonly used in agricultural interventions, did not maintain its initial colonization on the roots of *Arabidopsis thaliana* following transfer to a new growth medium in a hydroponic system. We hypothesized that bacteria native to the *A. thaliana* rhizosphere might be able to increase the abundance of *B. subtilis* cells associated with plant roots over time. By designing a unique experimental system to study bacterial colonization and maintenance of hydroponically grown plants, we successfully identified three bacterial isolates that increased abundance of three PGPB *Bacillus* strains at initial colonization and subsequent growth in a minimal carbon medium. Here, I summarize the potential applications and need for these experimental approaches to fill gaps in our knowledge and drive future research in the field.

In the introduction to my dissertation (Chapter 1), I reviewed the current literature relevant to rhizosphere colonization and maintenance by *Bacillus* species. I described the demonstrated beneficial effects of *Bacillus* PGPB on plants as well as their interactions with plants over time. After summarizing the key mechanisms by which *Bacillus* spp. increase plant growth and development, I described the various methods for studying PGPB-plant interactions

currently in use. I particularly highlighted studies of *Bacillus* colonization and maintenance of plants and discussed the factors that may contribute to the discrepancies of PGPB treatment efficacy observed between laboratory experiments and those conducted in the field.

In Chapter 2, I described the creation of an experimental system to study bacterial associations with plant roots following transfer of the host plant out of the initial environment of bacterial colonization in a hydroponic system. Specifically, I asked about the association of bacteria with plant roots after their initial co-incubation in a carbon-rich hydroponic condition and how they maintained that association following transfer of the plant into fresh, minimal carbon (plant-optimized), liquid growth medium. We designed this system to allow us to quantify CFU of bacterial strains, whether in mono-association with the plant host or as part of a multispecies bacterial community, and to describe the abundance and localization of fluorescent bacteria along the root using microscopy. We first verified the utility of this system for studying PGPB-plant root colonization through experimentation with a PGPB strain of *P. simiae.* We then tested the colonization kinetics of three bacterial isolates found in the *A. thaliana* rhizosphere, finding that each individual isolate maintained initial colonization whether on its own and or in combination with the other two bacteria. We found that one of the bacterial strains can not only maintained initial colonization, but also increased in abundance, and that mixed bacterial species co-colonized and maintained associations with the plant root over time. This experimental system facilitated our own subsequent work (Chapter 3), and we hope that sharing this method through the publication of a video protocol will also prove useful for other researchers within our field.

PGPB strains of *Bacillus* that were unable to maintain initial root colonization on their own demonstrated increased colonization and maintenance when co-inoculated with other

rhizospheric bacteria. We demonstrated that our new hydroponic assay (Chapter 2) was beneficial for examining microbial behaviors in the rhizosphere, both for studying the maintenance of root colonization by undomesticated bacterial isolates and for measuring the root colonization kinetics of PGPB strains within bacterial multispecies communities. We characterized a collection of 96 fully genome-sequenced isolates (from the rhizosphere of *A. thaliana* grown in native soils) for their ability to maintain root colonization, and found that 61% of isolates could maintain colonization when in mono-association with the plant root. We then screened this collection for strains that could increase the colonization and subsequent maintenance of *B. subtilis*, which only poorly associated with the *A. thaliana* root as a monoculture. After rescreening our initial hits in a secondary screen, we then narrowed the number of potential 'helper' strains to three bacterial isolates: an *Agrobacterium,* a *Variovorax,* and a *Methylobacterium* species. By extending the length of our maintenance phase of incubation (from one to three days), we also measured the kinetics of *B. subtilis* root association in combination with these species. Microscopy imaging showed that these three helpers affected the abundance and localization of *B. subtilis* along the root and indicated that they were in direct contact with *B. subtilis* cells during co-colonization. Further, we showed that performing this screen using the highly tractable model organism *B. subtilis* NCIB3610 could be valuable for identifying stains that were similarly beneficial for other agriculturally relevant *Bacillus* species (*B. amyloliquifaciens* spp. GB03 and FZB42). Interestingly, we found no indication of additive or detrimental effects from using a combination of the three helper strains relative to using them individually.

4.2. Significance

PGPB-based products are marketed as the future of agriculture: ideally, they would allow farmers to produce more crops with less consumption of environmentally and fiscally expensive chemical measures[1–4]. Reaching these goals would be facilitated by a better understanding of the complex interkingdom interactions occurring within the crop rhizosphere. Dissecting communities for reproducible behaviors should allow researchers to predictively formulate sustainable bacterial communities to benefit crop growth, yield, or pathogen resistance based on the host plant or its environment.

Although historically studied in monoculture, bacteria are now understood to exist in complex multispecies communities which display emerging properties unique to the community[5]. Due to recent advances in research technologies, scientists are increasingly turning towards studying the inherent intricacies of these bacterial communities [6,7]. The findings of this dissertation provide an important framework not only for designing methods to modulate such microbial communities, but also to understand the biological processes occurring in natural ecosystems. Broadening the knowledge base of how bacteria interact within multispecies communities in the presence of their eukaryotic hosts could ultimately allow researchers to directly manipulate these communities for benefit of the host. In the case of agricultural applications, these approaches could lead to improved development and implementation of PGPB-based interventions for increasing crop yields. In addition, our findings can be applied to bacterial interaction research across disciplines, just as research into the oral and gut microbiome has set standards for other bacterial community studies[8,9].

Bacteria quickly and effectively react to their environment by changing their behaviors based on chemical and physical inputs. It is unknown how physicochemical and biological

factors affect the survival or behaviors of PGPB; both the presence and activities of PGPB are considered likely to be important for their effective use as crop bioadditives. One phytosphere property that has not been fully explored for its effects on PGPB intervention efficacy is the endogenous microbial population. It is plausible that neighboring microbes could affect the growth or behaviors of PGPB, like *Bacillus* species[10,11], either to increase or decrease their beneficial effects on plant growth or development. By addressing this gap in knowledge about the role rhizosphere bacteria play in modulating the outcomes of PGPB, scientists and commercial product producers could better understand how to develop PGPB interventions effective across environments by considering the specific phytobiome of interest.

Towards these goals, my research produced important information about the differences in bacterial species' ability to colonize and maintain associations with the root of *A. thaliana* following transition to a different growth environment. Previous *in vitro* research with PGPB *Bacilli* has mainly focused on plant root attachment in conditions where carbon sources are readily available to allow growth of the bacteria within the nutrient medium itself[12,13]; this allows for fast a highly reproducible colonization of the host. Since soil contains much lower concentrations of simple sugars than these media[14], we wondered whether root attachment would be maintained once the plant, already colonized by bacteria, was transferred to a low-carbon nutrient medium where the plant would be providing more sugars than the surrounding liquid.

Existing literature indicates that *Bacilli* frequently do not maintain their initial root colonization levels over time[15,16], supporting our call for increased focus on prolonged plant-microbe interactions non-carbon-rich laboratory environments. We were not surprised to find that, in a more stringent growth environment, reproducibility of outcomes was lower than in reported findings from previous studies. We suspect that this is due to the inherent complexity of

studying interkingdom interactions, where each biological organism adds to the variability of measurements. While our experimental design was focused on reducing this variability (for instance, by choosing seedlings of approximately the same size and inoculating with cultures of exactly the same titers of bacteria across biological replicates), small differences in root structure could still affect the plant-microbe interactions. This biological complexity increases the number of samples needed to reach statistical significance; in some cases, clear trends were apparent but, statistical significance at our cutoff values was not reached. Future studies will need to account for this variability, which may be especially difficult in large-scale field environments. Nonetheless, these approaches are necessary to validate and identify the most robust effects, which will be those that are most likely to overcome the additional variability inherent in crop fields in open environments.

Producers of PGPB-based agricultural interventions promise increased crop yield in the face of many different stressors, from invasion by pathogens to cyclic flooding and drought[1–3,17]. Furthermore, the marketing behind these approaches often claims that the use of PGPB will decrease our reliance on chemical pesticides and antibiotics[4,18] while avoiding consumer concerns about using GMO crops[19]. While commercial products do indeed show great potential in fulfilling these assurances, the efficacy of treatments varies by ecosystem, plant species and ecotype, sampling type, and even scientific study[20–23]. Rather than accepting them at face-value and churning to blindly implement these measures, we should push back against these claims to understand where biology deviates from idealism to create even better solutions to today's agricultural challenges.

Only a fraction of soil-dwelling bacteria have been well studied due to challenges in culturing them and a dearth of genetic tools with which to examine them[24]. Our method of

screening the effects of uncharacterized (and likely genetically intractable) bacterial isolates allows for the exploration of these species' interactions within the rhizosphere environment. Our finding that only around 61% of tested rhizosphere bacteria could maintain an association with the same host plant from which they were originally isolated was curious. Our experimental conditions were clearly distinct from those of native soil, but these results provide additional reasons to not only examine the interactions between bacterial consortia and the plant root, but also support studies that explore a range of agriculturally relevant environments to which results are to be applied. For example, our methodology could be used to test the effects of common agrochemicals or environmental stressors likely to be encountered by bacteria within a host plant's rhizosphere. Following initial colonization, seedlings embedded in mesh could also be transferred to soil substrates or other growth systems.

Although my work focused on understanding interactions between well-studied model organisms (*B. subtilis* and *A. thaliana)*, we intentionally chose natural rhizosphere isolates[25,26] to ask whether some of these bacteria could promote the growth of commercially produced PGPB strains. This was based on existing literature indicating that growth within multispecies communities can increase the survival and proliferation of individual bacteria[7,27], as well as studies showing that soil-dwelling bacteria affect *B. subtilis* behaviors required for plant colonization[10,28,29]. Our new experimental system for use in studying plant-microbe interactions (Chapter 2) may be useful for future research in the field of multispecies bacterial interactions, as the protocol is easily amenable for use with many different species of bacteria, plants, and environmental conditions[30]. Our studies show that bacterial association with plant roots changes over time and conditions, suggesting that colonization and maintenance kinetics should more

commonly be investigated; this is especially true when making claims about the long-term effects of PGPB-based interventions.

During the creation of our experimental hydroponic growth system, we found that the PGPB *P. simiae* and a select few rhizosphere isolates could maintain colonization following transfer to the new environment[30]. What is not apparent in the presentation of this work is that we first tested our system for use with *B. subtilis* NCIB 3610 and were dismayed to find that *B. subtilis* did not maintain colonization on roots, which was in opposition to reported findings of prolonged plant association and rhizosphere effects by different species of *Bacilli*[16,31–35] – was our methodology faulty?

Through discussions with colleagues, we discovered this "loss of colonization" phenomenon was often observed but not always explicitly discussed in publications[15,23,33]. Through re-examination of reported data, we realized that this effect has been underappreciated in the current literature. Throughout the process of collecting data and writing both the research manuscript and this dissertation, we have found more scientists who anecdotally confirm they have observed similar results. We hope this work promotes open discussion and dissection of PGPB research, even when findings challenge exciting hypotheses that gain interest, funding, and company support.

4.3. Future Directions

Continued studies on the mechanisms underpinning bacterial plant growth-promotion are essential for generating agricultural bioadditives, like those comprising PGPB *Bacillus* species, to increase plant yields and protect crops from the devastating impacts of pathogens[16,31,32]. Focusing on the colonization and maintenance patterns of rhizosphere bacteria, including

studying the spatiotemporal development of associated communities on roots in reductionist experimental approaches, will increase our understanding of the properties underlying these interkingdom interactions[26,33,36–39].

Scientists continue to develop novel experimental approaches to approximate different aspects of PGPB-relevant environments, including using transparent "pseudo-soils" and assays to assess specific functions of potential signaling molecules such as VOCs[39–41]. Adding external dyes or fluorescent probes can allow researchers to identify the localization of non-fluorescent strains through microscopy imaging[8,8,25]. Alternatively, researchers could focus on visualizing the spatiotemporal association by genetically tractable or naturally fluorescent strains for their interactions in these systems[36,42]. Similarly, differential selective plating can be adjusted to quantify bacterial CFU from different genera and species, similar to the selective plating used here to count *Bacillus* spp. CFU even when inoculated with other bacteria.

If the ultimate goal of PGPB research is to increase crop yields, it would be prudent for researchers to conduct experiments in complex systems that more closely approximate real-world conditions. As microbiome composition varies by host plant genetics and phenotypes[26,43], identification of additional PGPB under varying conditions should be expanded to larger numbers of relevant plant species[2,12,44]. Performing high-throughput screening of model organisms in defined environments could help to expedite the design of larger studies[30]. In addition, as sequencing technologies and additional biochemical tools become more affordable and widespread, and our knowledge more complete, a personal-medicine-type approach may enable improved fertility of both small-scale and industrial-sized farming fields[32,45–47].

Endogenous microbial communities inhabit all phytospheres, yet key questions remain about how these diverse microbiomes impact the capabilities of exogenous PGPB to exert

predictable impacts on plants[15,34,48,48]. Many of these microbes, whether plant pathogens or not, can inhibit the growth and development of *Bacillus* populations[46]. Understanding how environmental conditions and different nascent bacterial communities within fields impact PGPB survival and behaviors – and whether, in turn, PGPB exert lasting impacts on those communities– will be necessary for the formulation of effective bioadditives for use across diverse ecosystems. As the relative abundance of bacterial species in the rhizosphere changes over time[49], and the age and developmental stage of the plant can also affect microbiomes' compositions[45,50], ideally studies must be conducted over the entire time scale of the interventions; thus, beneficial effects should be monitored over the lifecycle of crop plants, from initial treatment to harvest. As PGPB formulations would preferably be effective across various conditions, some of which might not be conducive to the growth of particular microbial species, it would be advantageous to create consortia where only some of the bacteria would need to persist to elicit beneficial effects.

Academic and commercial labs alike are turning their focus towards identifying biological and chemical additives that confer robust benefits to the plant host's growth[7,35,44,47]. Should specific chemical compounds amplify or prolong the beneficial effects of PGPB treatments, genetic engineering may allow scientists to construct microbial inoculants capable of producing chemicals and metabolites not found in their native genomes[32]. Alternatively, these chemicals could be added directly to the growth substrates or even be expressed by genetically modified host plants.

PGPB-based agricultural technologies hold great promise for solving some of societies' most pressing problems, including famine and ecosystem destruction, while reducing the cost of chemical production and repeated application. However, manipulating biological systems is

incredibly complex and may result in unexpected and deleterious outcomes, such as depleting the diversity of ecosystems[39,45,48,51–53]. We must be careful in the marketing and implementation of emerging biological technologies, as has been recently illustrated by the public's response to Genetically Modified Organisms (GMOs). Although peer-reviewed studies suggest that consuming GMO foods does not disrupt human health, GMO crop products have faced resistance to the point where consumers purchase products based on the GMO-free label. This outcome has greatly limited the utility and potential of GMOs to address existing agricultural problems. Overselling the benefits or understating the uncertainties of emerging PGPB technologies could result in similar scenarios that restrict their broader implementation in global markets.

Continued work on the projects outlined in this dissertation may add to our understanding of interspecies and interkingdom interactions, both within and beyond the scope of phytobiome research. Findings from these studies can be used to design effective methods for studying multispecies bacterial activities across various settings, especially for the determining spatiotemporal kinetics of community behaviors. Their application to agricultural science might allow for more accurate assessment of the likely efficacy of PGPB-based treatments during changes in the phytosphere. Ideally, this research will guide future work in building multispecies bacterial communities to improve PGPB effects and subsequent crop yields. It is through the endeavors of future researchers that we will address the challenge of feeding a growing population in the face of rapid climate change[54].

4.4. Future Experiments

The following are suggested experiments to answer outstanding questions resulting from the work described in this dissertation:

Monitor spatiotemporal development rhizosphere communities

To monitor spatiotemporal development of rhizosphere communities under different conditions, colonized seedlings on floats could be transferred to maintenance environments of various chemical composition and physical structures. To understand how the nutrient and chemical composition of the maintenance environment affects community stability and composition, floats could be transferred to media with varying levels of carbon, salts, and osmolytes. To assess how physical structures and biotic components of soils affect community dynamics, floats could be transferred to sands or soils. These experiments could be conducted with or without the addition of other microbes, whether through inoculation of specific strains or bulk bacterial soil or rhizosphere isolates into the substrate, or by using a soil substrate containing endogenous soil microbes. Antibiotics or chemical additives could be added to measure effects of common agricultural interventions that might affect root-associated bacterial communities.

To observe changes in species composition and relative localization in a mixed community, researchers could monitor presence and localization of bacterial strains by measuring relative genetic material abundance via 16S rRNA amplicon sequencing[55] or visualizing species using microscopy. Using dyes[56,57], FISH probes[25], or genetic engineering for expression of fluorescence markers[58], studies could be designed to qualitatively and quantitatively measure the localization of multiple bacterial strains during association with the

plant root. Seedlings could be grown in systems where imaging can be performed without destructive sampling[36]. This would allow for more accurate measurement of colonization events, including whether bacteria move along the root once in association and by providing more discrete information about the kinetics of bacterial colonization and detachment.

Identify genes and processes of bacteria that increase PGPB colonization and maintenance

To understand which behaviors are important for the development of rhizosphere communities, researchers could measure bacterial gene expression during association with the plant root, especially for genes controlling behaviors known to be relevant to colonization and interspecies interactions. Examples of genes of interest (GOI) would be those required for biofilm formation, secondary metabolite production, and motility and chemotaxis. To indirectly monitor levels of gene expression, bacterial strains could be engineered to conditionally express fluorescent proteins under the control of GOI promoters. Transcriptional expression of GOI could be directly quantified by RT-PCR or RNA-sequencing[59]. To verify the importance of specific genes or metabolites during colonization and maintenance, genetically tractable strains could be mutated to delete genes and mutant bacterial strains could be compared with the parental strains for their ability to affect PGPB root associations.

Create PGPB consortia for use in agricultural settings

Researchers are seeking to create new consortia for use in varying environments or plant hosts; directed screening could speed this process. For example, researchers could use our experimental system to identify a bacterial consortium that promotes the maintenance of PGPB *Bacillus* species when treated with antibiotics or other chemical compounds used in agriculture.

Alternatively, species could be isolated from the rhizospheres of other plant hosts of interest that can be hydroponically grown and tested for their ability to promote PGPB association with that plant. Although we focused on identifying single strains that increase the maintenance of PGPB *Bacillus* species, microbes could also be screened in mixed-species combinations or researchers could look for bacteria that increase the plant association of other PGPB strains that are unable to maintain colonization on their own. Given a large enough selection of potential helper strains, comparative genomic approaches could be used to detect genes and biological pathways shared by identified such beneficial strains of bacteria[60.] Computational models based on identified strains and relationships could be designed to predict which consortia might produce desired effects[61].

To validate effects of helper strains on the prolonged association of PGPB with host plants of interest, defined consortia could be applied to crop plants in field settings and PGPB strains' abundance quantified over the course of a typical growth cycle. These would be conducted in the environmental settings most similar to those of commercial farms growing the specific crop plant. A major fault of many existing studies is that they neglect to take initial samples (prior to inoculation of the bacterial strains of interest) to assess the baseline endogenous levels of bacteria of interest or to monitor species-level abundance[62]; we propose researchers should ascertain whether their specific strain maintains plant association and/or presence in the surrounding soil throughout their studies and whether the titers found in subsequent samplings are higher compared to before the initial introduction of that strain.

To validate the underlying assumption that an increase in PGPB *Bacillus* presence leads to increased PGPB-related effects on plant growth, measurements of plant growth and development should be taken over the lifespan of the host plant when initially colonized by: the

PGPB plus helper bacterial strains, the PGPB only, the bacterial helper strains only, and without any colonization. Measurement sampling might include shoot or root size, root structure, fruit size and production, resistance to drought or pathogen stress, or other relevant plant characteristics.

4.5. Closing Statement

Scientists are driven by a desire to understand and even improve the world through logic, observation, and critical thinking. As biologists, we take rigorous measures to dissect, compare, and model how the living world appears to operate. Our projects rely on the integrity and thoroughness of those conducted before us, and we aim to create new knowledge to guide the researchers who pick up where we leave off. In conclusion, I am pleased that this dissertation fulfils the requirements of providing valuable information to basic and translational fields of research; however, I am more so enthused that these findings may contribute to the development of crop production interventions with a lowered environmental cost and may ultimately benefit the future of our planet.

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APPENDIX: GET ONLINE TO SUPPORT WELLBEING OF GRADUATE STUDENTS

A.1. Overview

Universities should use online resources to help graduate students who are struggling with their mental health to access appropriate support. This Feature Article is part of a collection on Mental Health in Academia.

A.2. Introduction

Mental health issues are a serious problem in academia, especially among graduate students where around 25–40% face mental health concerns[1–3]. Moreover, about half of those students will likely not receive treatment[4]. These figures highlight the pressing need for the academic community to both offer more support for graduate students struggling with their mental health and remove the barriers that prevent those students from getting help where it already exists.

We are two PhD students at universities in the United States. We both also volunteer with PhD Balance[5], an online community dedicated to sharing resources and stories to empower graduate students professionally and personally. Susanna founded PhD Balance[5] in 2018 (originally called "The PhDepression") to find other students managing mental illness while in graduate school. Liesl joined after her own mental health experiences lead her to reach out for the resources and support provided by groups like PhD Balance[5]. At the time of writing, the PhD Balance[5] community includes over 36,000 followers, across Twitter[6] and Instagram[7], from six continents, and these numbers continue to grow.

In this article, we highlight factors that may prevent graduate students from accessing mental health support as it exists now, before exploring how online tools can overcome barriers to access with examples drawn from personal experiences within the education system in the United States. Finally, we recommend concrete steps that institutions can take to better support the mental wellbeing of graduate students via online resources.

A.3. Existing Support Options

The factors affecting the mental health of graduate students are complex, and each student's experience is unique. Yet, based on conversations with thousands of graduate trainees and other academics through PhD Balanc**e**[5], we have identified some common themes that are often reported.

The transition from undergraduate to graduate studies can lead many students to shift how they view the world and how they perceive themselves in it[8]. This potentially jarring and stressful experience can lead students to question their aptitude for the field. This, in turn, may reduce their likelihood of acknowledging signs or symptoms of mental health distress and seeking help[9]. Additionally, graduate students often feel guided, either explicitly or implicitly, to emulate their mentors[8]. If a student is told to get rest or spend time with friends but sees their mentor often working late and seemingly neglecting other responsibilities, the student is more likely to mirror the behavior than take the advice. Further, PhD advisors may also repeat the behavior of others, potentially leading to academic "hazing". This occurs when a mentor or superior challenges a new student to essentially 'prove themselves worthy' because that is how they were treated when they entered the field[10], even though the pressure can be harmful to the mental wellbeing and productivity of the student. Lastly, through experience we know that

graduate school can be isolating for a number of reasons. When a student is struggling, they may not know how to get help, and if a student feels isolated and alone, it only serves to make the struggle worse.

Though not all students struggling with their mental health will seek help from their university, institutions of higher education have reported a nearly 30% increase in the use of university mental health resources between 2010 and 2015[11].Some institutions, for example, offer courses on time-management and work/life balance, or even classes in meditation or yoga; however, such activities appear to, at best, offer short-term benefits and are often ill-attended by graduate students[8,12].

Most universities list their in-person resources on their websites, allowing students to access some basic information without directly contacting a university employee. However, there is a lack of data around which universities supplement these offerings through the curation of more detailed web pages or access to external online resources. One example of an online resource being leveraged by a university is the "WellTrack" web app[13] at Purdue University, which allows students to track and monitor their own mental health and provides coping and mitigation methods to the students separate from the university's counseling program. It would be useful to know whether online interventions are being implemented more widely and how effective they are in supporting both undergraduate and graduate students.

One common issue we have encountered with mental health interventions at universities is that they are almost always tailored to undergraduates, because undergraduates typically make up the bulk of the student population on any campus. However, factors such as housing, finances and stage of life will vary greatly between these two groups[14]. The stories collected through PhD Balance[5] show that a graduate student's experiences are often unlike those of undergraduates

studying at the same university but share similarities with PhD students across institutions and topics of study.

We believe that universities need to shift the focus of at least some of their mental health provision to better serve graduate students. We are aware that offering more nuanced resources may pose a major challenge for university administrations facing budgetary and regulatory restrictions. However, while they cannot be replacements for in-person interventions, we believe that online resources may be a convenient way for graduate students to seek help that is more tailored to their unique needs.

A.4. Benefits of Online Resources

Timely response to a mental health crisis can be the difference between life and death. Students may feel uncomfortable contacting emergency medical services, such as calling 911 in the United States. They may also not be financially capable of using these emergency services if there is a charge, because money concerns are common problems faced by graduate students[15]. Around-the-clock access to responsive providers may be critical in supporting students outside of regular academic hours. Suicide rates peak in the spring regardless of hemisphere[16], which is a transition time for universities as they prepare for the end of the academic year – meaning that students may unfortunately also experience lapses in access to student health services at this time. One solution to partially address these concerns is for institutions to clearly list crisis hotlines, both local and national, and other free resources prominently on their websites.

Thoughtful use of websites and digital media could also help in guiding users to relevant resources, whether housed online or available in person or via phone. Online resources may complement existing systems; for example, if a student is concerned about their mental health,

they can use the online version of the Patient Health Questionnaire[17] (i.e., PHQ-9) to assess their indicators for anxiety and depression. While the results of this evaluation would need to be verified by a certified mental health provider, the student may feel more comfortable with and capable of reaching out to university mental care facilities when they are already equipped with this information.

Though there are a number of movements to "end the stigma", negative perspectives of mental illness are pervasive in academia[18]. The fear of facing such stigma, for example from their supervisor or peers, can pose additional hurdles to a student in need of assistance[8]. Access to online resources offers anonymity, which can bypass those barriers to accessing support.

When a student expresses concerns about their own mental health, loved ones and colleagues alike can benefit from being able to quickly access the university's resources online. If the student has moved away to university, guidance from their supervisor and peers can be especially important and feeling supported by a mentor has been cited as a crucial indicator for a graduate student completing their training[15]. Resources that are accessible online can help advisers to learn about current issues and provide informal mental health support for their trainees without necessarily taking additional training.

Online platforms have the power to connect people to form communities even if they are separated by geography. By providing a community where everyone can share their own experiences, it helps others know they are not alone in their experiences. This sense of community and shared experience can assist in decreasing stress and lowering the sense of "hopelessness" in an individual's own struggle. PhD Balance[5] provides this community through sharing member-submitted and curated stories. Additionally, it gives a space for people to discuss their own struggles and crowd-source advice through several social media outlets.

With an estimated one mental health specialist for every 1,700 students, front-line support – like student services offices – is often created to serve the "average" student demographic[4]; however, members of different populations require different interventions, which could be supplemented through providing online resources. Minorities often face additional challenges related to a feeling of "otherness" and a lack of access to resources[14]. Underrepresented minority (URM) students are less likely to have adequate mentorship and personal support, potentially limiting their success[15], and international students are less likely to use mental health services[19]. Web pages can be translated into various languages to allow students and their families to gain access to resources even if no one in the human support services is fluent in their primary language. Lastly, people with disabilities can also benefit from the use of online resources that are optimized for accessibility; students with mobility issues do not have to visit an in-person facility, screen-readers provide information to those with vision impairment, and web pages can be easily read by people with a hearing impairment.

Many online resources created and optimized by larger institutions could likely be amended to fit the needs of a new university more quickly than changing in-person systems. Rather than recreating the wheel, universities might be able to model their own sites on others or even share tools and strategies. Updates to reflect changes in needs, policies or best practices can also be made immediately for online resources. One institution that can be used as a model for easy access to mental health services and online resources is the University of Michigan and its associated Rackham Graduate School.

Initial online searches (within the United States) for mental health resources will route you to the sites for the National Institute for Mental Health[20], the Substance Abuse and Mental Health Services Administration[21], National Alliance on Mental Illness[22], and other similar

national organization websites. These sites direct users to 24-hour helplines, crisis resources and resources to find health-care providers. Another search hit lists "80 Awesome Mental Health Resources When You Can't Afford a Therapist" and includes online forums, places to meet support groups, and apps that can help with guided meditation, online therapy sessions and self-guided behavioral therapy techniques[23]. Other online support systems tailored to graduate students, such as Beyond the Professoriate[24] and PhD Balance[5], can help students succeed both in terms of personal and professional growth, and there are many blogs focused on these topics too. Identifying and providing access to these types of resources can supplement university-specific initiatives and guide future resource creation.

A.5. Limitations of Online Resources

While we think there is a strong case for universities using online tools to help the mental wellbeing of their graduate students, inappropriate use or implementation of such resources can result in negative consequences. Institutions need to remain aware of their limitations. Firstly, these measures should never be used as a replacement for other types of existing services. Online resources may provide users with a false sense of resolution, and psychoanalyst and psychiatrist Mary Davis warns that reliance solely upon online interventions, including "meeting" with a teletherapist, may not be sufficient to address mental health issues if the patient does not open up as readily in an online setting[25]. Instead, rather than considering online services to be a solution, they may be better seen as temporary stopgaps and pathways to other measures that will see people receiving appropriate support.

Established online resources must also be maintained to ensure that are easy to navigate and that students do not face sudden barriers to care due to broken or missing links. Further,

separate departments and colleges within a university system must coordinate to keep up with changes in resources between web pages, which may require universities to dedicate money and time to maintain their digital resources.

Although most of us are reading this article via the web, internet access must not be taken for granted. About 10% of Americans do not use the internet (Perrin and Kumar, 2019)[26], and while graduate students do not necessarily reflect the overall population, many in their support networks still rely on phone or in-person routes to get resources. To address this, resources created on the internet can be modified to suit print dissemination (and vice-versa) to enable students of all backgrounds to access support.

A.6. Recommendations

We would make the following recommendations for universities looking to support the mental wellbeing of their graduate students through the curation of online resources. First, all resources should originate from one centralized location, allowing students to find them with minimal 'clicks' from the same starting location instead of using general search engines. Second, university systems already in place for undergraduate and professional school students (like medical students) could guide the organization and content of those tailored for graduate students, including the ways in which they are often sponsored through university funds. For instance, graduate schools could take inspiration from the Wellbeing Index for Physicians[27], which was created by the Mayo Clinic and sanctioned by the American Medical Association and allows doctors and medical students to evaluate their mental health over time and find local and national resources applicable to their current situation. Third, online resources should be accompanied by contact information with appropriate human resources so the user can access

further assistance. Finally, institutions can gauge the efficacy of online support initiatives through collection of metrics, both to improve the systems and to provide evidence for their continued support. Some metrics to be considered are the number of students who access the online resources; whether these website interactions lead students to contact appropriate support offices; and if students report awareness of and satisfaction with the online content.

A.7. Conclusions

We have seen firsthand how online resources can help graduate students struggling with their mental health. We would like to see more institutions deploy them as part of their wider provisions to support mental wellbeing of their different student populations. We hope that their potential to remove the barriers that may limit current access to appropriate support will mean that no graduate student is left struggling without help.

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